The role of nonspecific cytotoxic cells and cytokine production in the pathogenesis of human intestinal diseases

by

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STATEMENT

All the experimental work reported in this thesis was performed entirely by the author except for the immunohistochemical studies (Chapter 2) and chromatographic experiments (Chapters 7 and 8) where assistance was provided by Dr. W Allan and Ms. J Hargreaves respectively.

The scientific papers cited in the introduction and discussion sections of this thesis were reviewed in the literature reported up to and including the month of August, 1987.

The protocols used in this thesis were approved on the 19th of July, 1984, by the Clinical Research Committee of the John Curtin School of Medical Research, Australian National University.

P.G. Hogan

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APPENDIX A

A number of manuscripts, either accepted or submitted for publication, have evolved from the data presented in this thesis. A list of these papers is shown below.

Hogan PG, Hapel AJ, Doe WF. Lymphokine-activated and natural killer cell activity in human intestinal mucosa. J Immunol 1985;135:1731-8.

Hogan PG, Basten A. What are killer cells and what do they do? Blood Reviews (accepted for publication).

Hume DA, Allan W, Hogan PG, Doe WF. Immunohistochemical characteristics of macrophages in human liver and gastrointestinal tract: expression of CD4, HLA-DR, OKM1 and the mature macrophage marker 25F9 in normal and diseased tissue. J Leucocyte Biol 1987;42:474-84.

Hogan PG, Gibson PR, Hapel AJ, Doe WF. Intestinal lymphokine-activated killer cells in inflammatory bowel disease. (submitted).

Hogan PG, Gibson PR, Hapel AJ, Doe WF. Intestinal lymphokine-activated killer (LAK) cells - cytotoxicity for colon cancer cells and modulation of their generation and cytotoxic activity. (submitted).

Hogan PG, Hapel AJ, Doe WF. Intestinal lymphokine-activated killer (LAK) cells-cytotoxicity for colon cancer cells and modulation of their generation (abstr). Gastroenterology 1986;90:1462.

Hume DA, Allan W, Hogan PG, Doe WF. Immunohistochemical characterisation of macrophages of the intestinal mucosa using the mature macrophage antigen 25F9 (abstr). Gastroenterology 1986;90:1469.

APPENDIX B

Cell surface antigens mentioned frequently in the text are designated by 'cluster-defined' (CD) nomenclature according to the Third International Workshop on Leucocyte Typing (McMichael AJ (ed), Leucocyte Typing III White Cell Differentiation Antigens, Oxford University Press, Oxford, 1987, Appendix E).

The CD designations used in this thesis are shown below:

CD2 ..... sheep erythrocyte receptor (OKT11, Leu-5).

CD3 ..... T cell antigen receptor-associated activation complex (OKT3, Leu-4).

CD4 ..... T cell helper/inducer subset (OKT4, Leu-3).

CD7 ..... all T cells and thymocytes.

CD8 ..... T cell cytotoxic/suppressor subset (OKT8, Leu-2).

CD16 ..... Fc receptor for IgG (Leu-11).

CD20 ..... B cells.

Surface antigens not referred to by CD nomenclature include:

OKM1 ..... monocytes, neutrophils, natural killer cells.

Leu-7 ..... natural killer cells, some T cells.

Leu-19 ..... natural killer cells, lymphokine-activated killer cells some T cells.

Ia ..... class II MHC antigens.

ABSTRACT

Colon carcinoma and inflammatory bowel disease (IBD) are common and important intestinal diseases where primary abnormalities of the local mucosal immune system may be of pathogenic significance. Nonspecific cytotoxic cells such as natural killer (NK) and lymphokine-activated killer (LAK) cells may have an antineoplastic role in colon carcinoma and may contribute to mucosal injury in IBD. The production of cytokines locally in the intestinal mucosa may be important for the maintenance of cytotoxic mechanisms and may have proinflammatory effects in IBD. The aim of this thesis therefore, was to investigate the role of mucosal nonspecific cytotoxicity and cytokine production in the pathogenesis of colon carcinoma and IBD. This question was approached by measuring and comparing levels of cytotoxicity in specimens of histologically normal intestine resected for colon carcinoma or nonmalignant disorders and actively inflamed mucosa resected for IBD. A possible relation between interleukin 2 (IL-2) and colony stimulating factor (CSF) activity and colon carcinoma was explored by assaying the levels of these cytokines produced by histologically normal specimens of mucosa resected for carcinoma or nonmalignant conditions.

The frequency and distribution of intestinal NK cells was first studied by immunohistochemical examination of mucosal lymphoid tissue for cells bearing the NK cell-associated Leu-7 surface antigen.

Functional studies were performed on local mucosal populations of

lamina propria mononuclear cells (LPMC) and peripheral blood mononuclear cells (PBMC), which were assayed for spontaneous and interferon (IFN)-inducible NK cell activity, LAK cell activity and antibody-dependent cellular cytotoxicity (ADCC). Lymphokine-activated killer cells were generated from LPMC precursor cells in vitro, by culture in the presence of interleukin 2 (IL-2). In order to optimize the levels of cytotoxicity obtained, mononuclear cells were enriched for NK cells and LAK precursor cells by density gradient centrifugation over Percoll. In addition to the standard cell lines used to test for NK activity and ADCC, freshly isolated colon carcinoma cells and intestinal fibroblasts served as more biologically relevant targets for intestinal NK and LAK cells. Mononuclear cell populations were also examined for their ability to produce IL-2 and CSF activity, either spontaneously in culture, or after stimulation with phytohaemagglutinin (PHA).

Lamina propria mononuclear cell populations displayed very low or undetectable levels of spontaneous and IFN-inducible NK activity against K562 targets and minimal ADCC was also registered against antibody-labelled P815 cells. There was also no appreciable spontaneous mucosal cytotoxicity directed towards freshly isolated colon carcinoma cells or intestinal fibroblasts. This paucity of NK activity was consistent with the very small numbers of Leu-7^+ cells revealed in mucosal sections by immunohistochemistry. The numbers of mucosal NK cells and their minimal or absent levels of cytotoxicity against any of the targets tested, were comparable in all specimens, irrespective of the underlying pathology.

In contrast to mucosal NK activity, LPMC displayed potent LAK

activity after in vitro culture in the presence of IL-2. There was no difference between the levels of LAK activity against K562 cells generated from colon carcinoma, nonmalignant or IBD specimens, implying that there was no deficiency of LAK precursor cells associated with malignancy. Intestinal LAK cells had a wide spectrum of cytotoxic activity, lysing tumour cell lines, freshly isolated colon carcinoma cells and intestinal fibroblasts, all of which were resistant to peripheral blood NK cells. Intestinal LAK cells were also capable of ADCC, although they apparently lacked a $CD16^+$ Fc receptor. The presence of LAK cells active against colon carcinoma was not restricted to specimens affected by carcinoma but could also be induced in LPMC populations from IBD and in PBMC from normal controls.

Phenotypic analysis of intestinal LAK cells cytotoxic for K562 and colon carcinoma targets showed that both the precursor and effector cells were $CD2^+3^-4^-8^-16^-$ Leu-7^-. Intestinal LAK cells mediating ADCC were predominantly of the same phenotype, although a small proportion of the effector cells were $CD16^+$. The generation of intestinal LAK cells in vitro was totally dependent upon the presence of IL-2 and also required cellular proliferation. The process was sensitive to inhibition by low concentrations of hydrocortisone but was unaffected by cyclosporin.

Lamina propria mononuclear cells released biologically significant amounts of both IL-2 and CSF activity after stimulation with PHA, but only CSF was released spontaneously in detectable quantities. The amounts of IL-2 released from specimens resected for carcinoma were significantly reduced when compared to nonmalignant specimens, but the CSF activity of

PHA-stimulated LPMC was unaffected by the presence of carcinoma.

From the studies of mucosal cytotoxicity it can be concluded that NK cell-mediated cytotoxicity is unlikely to have a significant antineoplastic influence in colon carcinoma or an important role in mucosal injury in IBD. The presence of significant NK activity in some LPMC populations however, argues in favour of the existence of a very small population of NK cells, but the function of these cells is unlikely to be mediated through cytotoxicity. On the other hand, the presence of LAK precursor cells within almost all LPMC populations indicates that high levels of LAK activity are potentially available to the intestinal mucosa. The capacity of intestinal LAK cells to lyse NK cell-resistant colon carcinoma cells in vitro suggests that LAK cells may be effective antineoplastic cells in vivo. The lysis of epithelial tumour cells and fibroblasts of intestinal origin also raises the possibility that LAK cells may damage nonmalignant cells in the mucosa affected by IBD.

The ability of LPMC to secrete IL-2 and CSF in vitro suggests that the production of these lymphokines in the mucosal microenviroment may be important under both normal and pathological circumstances. The potential availability of IL-2 within the mucosa suggests that LAK cells may be generated from precursor cells in vivo under appropriate conditions such as chronic immune stimulation. The reduced level of PHA-induced IL-2 secretion associated with colon carcinoma may result in impaired local cytotoxic mechanisms that could otherwise be operative against the tumour. Abnormalities of IL-2 or CSF secretion may have other important consequences, particularly with respect to the initiation or prolongation of

mucosal inflammation in IBD.

The most important implication arising from the data presented in this thesis is that the intestinal mucosa contains both the appropriate precursor cells and secretory capacity to generate highly cytotoxic LAK cells, which may either protect or damage the intestine, depending on the nature and strength of the stimulus.

CHAPTER 1

General Introduction

Although it is now 14 years since the initial descriptions of natural killer (NK) cells, widespread interest in the origin and function of this cell lineage continues (1). Natural killer cells and other nonspecific cytotoxic cells such as the lymphokine-activated killer (LAK) cell are still under evaluation as potentially important antineoplastic, antimicrobial and immunoregulatory cells. This literature review presents an account of the nature and functions of human peripheral blood and intestinal NK and LAK cells. The potential involvement of NK or LAK cells in human disease is emphasized.

1.1 Characteristics of natural killer and killer cells.

Before the advent of surface marker analysis, NK cells were first recognized and then defined by their ability to lyse both autologous and allogeneic tumour cells (2,3,4). Although this operational definition is still widely used, it is now recognised that NK cells are a heterogeneous group with diverse functional and phenotypic properties (5). The cytotoxic activity of NK cells operates on first exposure to target cells and is independent of both extrinsic antigenic stimulation and the major histocompatibility complex. Cell lines susceptible to NK cells are derived

from leukaemias, B and T cell lymphomas and solid tumours including malignant melanoma and carcinomas of the bronchus, breast and bladder (4, 6-10). Malignant cells freshly isolated from resected tumours were regarded by many authors to be resistant to NK cell activity (11). However by using highly purified NK cell populations it has been shown that resting NK cells may display low to moderate levels of cytotoxicity against freshly isolated tumour cells from lymphomas and some carcinomas (12,13).

Natural killer (NK) cells constitute a unique lineage of large granular lymphocytes (LGL) which are postulated to arise from precursors in the bone marrow (14). Approximately 10-15% of peripheral blood lymphocytes (PBL) are LGL and of these 70-80% have the potential to act as NK cells (15,16). The remainder of the LGL pool lack NK cell capability and consist predominantly of T cells which mediate ADCC (17). All LGL have an indented, eccentric nucleus containing dispersed chromatin and an occasional nucleolus. The cytoplasm is plentiful and contains a prominent perinuclear Golgi apparatus. Azurophilic granules with the enzymatic profile of lysosomes (positive staining with acid phosphatase, β-glucuronidase and α-napthyl acetate esterase) are scattered throughout the cytoplasm (18,19,20). In ultrastructural studies, mature granules contain a uniform electron-dense matrix bounded by a unit membrane. Despite superficial similiarities to the monocyte, NK cells are myeloperoxidase-negative and nonphagocytic. Although generally regarded as lacking adherent properties, NK cells may adhere transiently to plastic

after initiation of culture (21). There is also evidence to suggest that LGL may be motile cells capable of chemotaxin-induced migration (22). Little is known about the traffic and possible recirculation of NK cells. LGL may possess specific homing receptors since adoptive transfer experiments have shown that infused T cells and LGL display different patterns of organ distribution in mice (23,24). The thoracic duct lymph is devoid of NK cell activity and therefore does not appear to be a major site for NK cells from the periphery to re-enter into the circulation.

LGL can be efficiently isolated from lymphoid cell populations by a method exploiting their relatively low density compared with mature resting T cells. Centrifugation over discontinuous gradients of Percoll separates a population of low buoyant density lymphocytes which are enriched for LGL (25). In the human, small numbers of NK cells are detectable in the bone marrow, spleen and lung but are suprisingly rare in the extensive mucosal surface of the intestine (20,26,27).

When only B and T cells could be identified by surface markers NK cells were considered to constitute the major "null" or "third population" cell in the peripheral blood. Recent phenotypic studies now reveal that NK cells express an array of surface antigens (28-39). Studies of this kind show that NK cells consist of multiple subgroups of cells which carry complex and often overlapping profiles of surface antigens. The finding of phenotypic heterogeneity is supported by cold target inhibition and absorption experiments which also demonstrate multiple NK cell subpopulations with exclusive or shared target specificities (31,32). The

diverse but overlapping target cell repetoires of cloned NK cells further illustrate this point (33).

The heterogeneity of NK cells has complicated their complete characterization by phenotypic or functional means. No single method of characterization can fully define the relative cytotoxic activity, frequency and target specificity of each NK cell type and furthermore, results depend upon the purity of the cell population studied. Depletion procedures using monoclonal antibody and complement-mediated lysis indicate only the proportion of NK activity mediated by cells carrying a particular surface antigen. On the other hand, multiparameter analysis of cells sorted by flow cytometry or immunoabsorption allows complete characterization of particular NK cell subpopulations and also represents a more sensitive method for detecting minority populations. As a group, NK cells active against K562 targets are predominantly $CD3^{-}2^{+}16^{+}$ $OKM1^{+}$ $Leu\text{-}7^{+}$ $Leu\text{-}19^{+}$ Ia^{+} (28,34-36). Most of these markers are not specific for NK cells and are shared with T cells (CD2, CD3, Leu-7), monocytes (OKM1) or neutrophils (OKM1, CD16). OKM1 and CD2 are present on 70-80% of K562-reactive NK cells and depletion of either surface marker decreases NK activity by approximately 80%. The CD8 antigen is present on 40% of NK cells but the relative contribution of these cells to total NK activity is controversial (35,36). The CD16 and Leu-19 antigens are found on the great majority of cells with NK activity while optimum treatment of peripheral blood lymphocytes with Leu-7 and complement deletes approximately 80-85% of total NK activity (28,29,37). The remaining

15-20% of NK activity is mediated by a potent minority population of $CD3^-16^+$ Leu-19^+ but Leu-7^- NK cells (27). A small component of peripheral blood NK activity is mediated by a completely separate lineage of $CD3^+16^-$ Leu-19^+ cells which also have the appearance of LGL. Variable proportions of these cells also express the CD8 and Leu-7 antigens. Some $CD3^+$ NK cells appear to utilize the T cell receptor (Ti)/CD3 complex to mediate their cytotoxic activity in a non-MHC-restricted manner (30,39,40). When considered as part of the entire NK cell population, these $CD3^+$ NK cells contribute 5% or less to the numbers of NK cells and their overall activity. The NK cell subsets which can be delineated using surface markers are shown in Table 1.1. CD16 is present on all $CD3^-$ NK cells except for a very small $CD3^-16^-$ Leu-19^+ subset (29). This small group of cells consists of both large granular and agranular lymphocytes which are proposed as possible precursors of mature NK cells. All NK cells, including the $CD3^+$ subgroup, express the Leu-19 antigen. Leu-19 is also carried by the majority of lymphokine-activated killer (LAK) cells, but has not been found on any other cell type so far studied (37,38).

Southern blot analysis of DNA from $CD3^-$ NK cells reveals germ-line configuration of the α,β and γ chain T cell receptor (Ti) genes whereas $CD3^+$ cells with NK activity possess the α and β rearrangements characteristic of mature T cells (39,41). Small numbers of $CD3^+4^-8^-16^+$ cells with an early form of the Ti coded for by γ genes also occur in the peripheral blood. As a group, these cells mediate ADCC but do not possess appreciable NK activity (17,42,43). On the basis of these findings, some investigators

Table 1.1 The surface phenotypes of peripheral blood natural killer cells.

Surface phenotype*	NK activity@
$CD2^{+}3^{-}16^{+}$ $Leu\text{-}7^{-}Leu\text{-}19^{+}$	+++
$CD2^{+}3^{-}16^{+}$ $Leu\text{-}7^{+}Leu\text{-}19^{+}$	++
$CD2^{+}3^{-}16^{-}Leu\text{-}19^{+}$	+
$CD2^{+}3^{+}16^{-}Leu\text{-}7^{+/-}Leu\text{-}19^{+}$	+

* Presence of surface antigen indicated by +; absence by -; +/- indicates the surface antigen may be either present or absent.

@ Comparative NK activity against the K562 cell line is graded from + to +++.

recommend that $CD3^+$ cells with NK activity should be classified as cytotoxic T cells (CTL) rather than NK cells.

1.2 Killer (K) cells and antibody-dependent cellular cytotoxicity.

Interaction between cell surface antigens and specific antibody may result in destruction of the cell by complement-mediated lysis, antibody-dependent cellular cytotoxicity (ADCC) or phagocytosis. At concentrations of cell-bound antibody too low to initiate complement activation or opsonization, ADCC remains an effective mechanism for causing cell lysis (44,45). In ADCC, antibody directed specifically towards the cell surface acts as a bridge between the target and the Fc receptor of the cytotoxic effector cell and by cross-linking Fc receptors the cytotoxic cell becomes activated and lyses the target (44). Cells capable of ADCC express Fc receptors for IgG and therefore include K cells, Tγ lymphocytes, monocytes/macrophages, eosinophils, neutrophils and LAK cells. The ability to mediate ADCC is an important facet of host defence against microbes and also possibly malignant transformation. Bacteria, fungi, protozoan and helminth parasites and viruses including the human immunodeficiency virus type 1 (HIV-1) appear to be susceptible to lymphocytes mediating ADCC (46-49). A special feature of murine intestinal lymphocytes is the expression of Fc receptors for IgA which facilitate ADCC of enteric pathogens recognized by specific secretory IgA (47).

The term Killer (K) cell refers to large granular lymphocytes which express the Fc receptor for IgG (the CD16 antigen) and are therefore capable of ADCC. The majority of the K cell population is phenotypically identical to $CD3^-$ NK cells and is regarded by many investigators as a subset of NK cells which have the additional capacity to mediate ADCC (15,50). Visualization of NK cells mixed with both NK-susceptible and antibody-labelled target cells confirms that a single NK cell can bind to and lyse both targets simultaneously (50). Furthermore, many cloned NK cell lines are capable of both forms of cytotoxic activity (51). Suggestions that separate cells mediated NK activity and ADCC were originally proposed on the basis of the differential effects of trypsin, interferon and prostaglandin E_1 on these forms of cytotoxicity (52,53). However these findings can also be explained by the differential effects of chemical agents on two separate classes of receptors on the same cell. The discordance between impaired NK activity and normal ADCC in the acquired immunodeficiency syndrome is also due to a selective defect of NK receptors which spares Fc receptors on the same cell (54).

A small proportion of K cells is comprised of $CD3^+16^+$ T cells which were previously designated as $T\gamma$ cells (17). These lymphocytes lack the CD4, CD8 and Leu-19 antigens and are incapable of NK activity. The existence of $CD3^+$ cells mediating ADCC has been questioned because depletion of $CD3^+$ lymphocytes from the peripheral blood does not alter ADCC (55). However direct phenotypic analysis of cytotoxic cells in ADCC plaque assays reveals $CD3^+$ lymphocytes in up to 40% of lytic conjugates.

The discrepancy between these findings may be explained by (i) the marked interindividual variation in Tγ cell numbers and (ii) a slower recycling time for Tγ cells compared to NK cells (17,55). The data suggest therefore, that that the more efficient and functionally important lymphocyte mediating peripheral blood ADCC is the NK cell.

1.3 The origin and differentiation of natural killer cells.

Sufficient evidence now exists to assign NK cells to a unique lineage of lymphocytes which arises directly from a multipotential stem cell. NK cells were originally thought to be derived from T lymphocytes or myelomonocytic cells because of their apparent expression of CD2, CD3 and OKM1 antigens during differentiation (56). However the existence of NK cells in invertebrates and in the human foetus well before T cells are detected argues in favour of a distinct NK lineage (57). The lack of Ti gene rearrangements in the majority of mature NK cells is further convincing evidence that they are not T cells (39). Apart from the common expression of OKM1, there are few fundamental similiarities between NK cells and the myeloid/monocytic lineages. The OKM1 antigen and other leucocyte function associated (LFA) molecules function as adhesion molecules for many cell types and are not lineage-specific (58). Analysis of human B and T cell immunodeficiencies supports the view that NK cells are derived from a common stem cell which also gives rise to granulocytes, B and T cells. The complete dissociation of NK activity and B cell function

in immunodeficiencies further suggests that the NK lineage is more closely related to the granulocyte and T cell precursors (59).

Abo et al have proposed a scheme for NK cell differentiation which is based on morphological, functional and phenotypic studies of NK cell populations in the lymphoid organs of foetal, neonatal and adult humans (20,40,56). The authors propose that NK cells at all stages of development are Leu-7$^+$. Immature cells in the bone marrow or peripheral blood showed increasing expression of the CD3 antigen, whereas mature cells lose CD3 and gain the CD16 and OKM1 antigens. Consistent with this scheme, the cytotoxic activity and granule content of the NK cells appeared to increase in parallel with the evolving surface antigen changes.

This scheme for the development of NK cells is under challenge, however, because of recent data. Detection of a functional Ti on a minor subset of CD3$^+$ cells with NK activity indicates that these cells belong to the T cell lineage and are therefore probably not an integral step in the differention of most NK cells as suggested (39). Furthermore, studies using more specific NK markers (CD16) indicate that the original scheme of differentiation proposed by Abo and Balch requires substantial modification. Although the majority of peripheral blood NK cells are CD16$^+$ Leu-7$^+$, a subgroup with the most potent NK activity lack the Leu-7 antigen (28). It is uncertain which subgroup is the more differentiated as both cell types have proliferative potential in short term cocultures with K562 tumour cells (60). In this system Leu-7$^+$ cells were not able to serve as precursors for Leu-7$^-$ cells. As CD16$^+$ Leu-7$^-$ cells can give rise

to either Leu-7 positive or negative cells they may occupy a more intermediate step in the differentiation pathway of NK cells (60). There is also disagreement in the literature concerning the expression of CD16 on immature NK cells in the foetus and neonate. Abo et al attributed significant CD16 reactivity in cord blood to immature granulocytes but independent observations by others suggest that $CD16^+$ LGL are plentiful in neonatal blood (61,62). The degree of expression of CD16 on NK cell precursors in the adult is uncertain but significant populations of $CD16^+$ lymphocytes have been detected in both the bone marrow and spleen (61). Although it remains to be proven, it is likely therefore that the CD16 antigen is present on all stages of developing NK cells. The Leu-19 marker is known to be expressed on both the $CD16^+$ and $CD3^+$ groups of NK cells in the peripheral blood, but similiar studies of other lymphoid compartments have not been reported (29). In addition, a small proportion of circulating NK cells are $CD3^-16^-Leu\text{-}19^+$ (28). The expression of the Leu-7 antigen on these cells has not been studied but it is postulated that this small population may be precursors of mature NK cells.

In the light of recent studies using new surface markers the original scheme of NK cell differentiation proposed by Abo et al should be abandoned. As yet the available data do not fit into a plausible scheme of NK cell differentiation but future proposals should acknowledge that $CD3^+$ NK cells belong to a lineage distinct from that of the bulk of $CD3^-$ NK cells. Further phenotypic and functional studies of human bone marrow and peripheral blood are required in order to identify early NK cell

precursors.

Human and murine studies provide circumstantial evidence that the NK progenitor cell is located in the bone marrow. Marrow transfer experiments in the murine system support this concept and also indicate that the microenviroment of the bone marrow is critical for NK cell development (63,64). Restoration of NK activity in severe human immunodeficiencies by bone marrow transplantation strongly suggests that NK precursors capable of repopulating the peripheral blood are located in the bone marrow (59). After bone marrow transplantation for human leukaemias, NK activity returns within 3-4 weeks, but it has not been convincingly shown that these cells are of donor origin (65). The generation of cells with NK activity from human or murine bone marrow in vitro presumably reflects the presence of NK precursors, but "contaminating" mature NK cells or LAK cells may also contribute (12,63). Little is known about the kinetics of NK cell differentiation. A clinical study of the effects of azathioprine suggests that the generation of significant numbers of functionally active NK cells may take up to 2-3 months, as this period of time elapsed after cessation of the drug before NK activity recovered completely (66).

1.4 Modulation of natural killer cell activity.

The importance of NK cells as antineoplastic cells has stimulated great interest in cytokines and pharmacological agents which can

potentiate or inhibit NK activity. Although the bulk of experiments on NK cell modulation have been performed in vitro, the effects of administration of recombinant interferons (IFN) and interleukin-2 (IL 2) in vivo are currently under investigation in clinical trials. This section deals primarily with the effects of IFN and IL-2 on NK cells in vitro, but other cytokines and mediators which influence NK cells are discussed. An account of attempts to modulate NK activity in vivo concludes this section.

Interleukin 2 is critical for the differentiation, cytotoxic activity and proliferation of human and murine NK cells. Functionally inactive foetal and neonatal NK cells can be stimulated to full cytotoxic potential by exposure to IL-2 (67). Short-term incubation with IL-2 for periods from 4 to18 h significantly boosts NK activity (68-70). This stimulation requires protein synthesis but is noted well before IL-2-induced proliferation is detected (71,72). Interleukin 2 also appears to maintain NK cells in their resting "physiological" state, because in the absence of IL-2, NK activity disappears within 20 h (72). This rapid boosting effect is unaffected by the anti-Tac monclonal antibody which blocks the proliferation of both T cells and LAK cells induced by exogenous IL-2 (28,69,71,74,75). The anti-Tac antibody reacts with a glycoprotein chain common to the high and low-affinity IL-2 receptors. The low affinity IL-2 receptor consists only of this chain but when it combies with another molecule to form a dimer, the high affinity IL-2 receptor is formed. These observations suggest that IL-2 may rapidly augment NK activity by

interacting with a receptor not recognized by anti-Tac. Alternatively, the recombinant IL-2 added to the cultures may successfully compete with anti-Tac for receptor binding sites.

Exposure of pure populations of mature NK cells to IL-2 in culture for periods longer than 24 h induces proliferation (68-79). The proliferation of NK cells requires only the presence of IL-2, but the response can be augmented by mitogenic lectins, interleukin 1 (IL-1) and IFN-γ (69,75,80). The optimal concentration of IL-2 for NK cell proliferation is tenfold higher than that required by cytotoxic T cells and kinetic studies detect NK cell proliferation occurring by 4 days in culture with peak activity at 6 days. The precursor frequency of proliferating NK cells in peripheral blood mononuclear cell (PBMC) populations was 1.4%, with a marginally lower NK cell cytotoxic precursor frequency (75). When cultured in the presence of IL-2, NK cells develop augmented cytotoxic activity and express increased densities of surface activation antigens (T10, Ia, IL-2 and transferrin receptors) (78). Unlike the rapid augmentation of NK activity, the IL-2-induced proliferation of NK cells is blocked by the anti-Tac antibody, indicating the participation of IL-2 receptors (68,70). It is uncertain how IL-2 can initiate NK cell proliferation in view of the very low numbers of IL-2 receptors on these cells (70,80). It is probable that sufficient numbers of high affinity receptors exist to trigger the division of NK cells. Under the stimulus of IL-2, NK cells may release lymphokines such as IL-1, IFN-γ and tumour necrosis factor (TNF), which singly or synergistically may upregulate IL-2

receptor expression, thus perpetuating NK cell division (80-83). Whether activated by IL-2 in a proliferation-dependent or independent manner, NK cells lyse a wide range of tumour cell lines and freshly isolated tumour cells which are normally resistant to resting NK cells (68,72,79).

Although IL-2 appears to boost both NK cell proliferation and cytotoxicity directly, it has been postulated that IFN-γ released by IL-2 stimulated NK cells may also be involved (69,84). Most authors discount a role for IFN in the rapid boost in NK activity induced by IL-2 because IFN-neutralizing antibodies have no effect on this process (68,72). However in some experiments, anti-IFN antibodies were inhibitory and so it cannot be excluded that small amounts of IFN in microenviroments protected from antibodies stimulate NK cells directly (69,70). It is generally accepted that IFN plays no direct role in NK cell proliferation, but it may be possible to 'prime' NK cells for the stimulatory effect of IL-2 by preincubation with IFN (69). Thus NK cell proliferation can be enhanced by the presence of IFN-γ, possibly due to an upregulation of IL-2 receptors (83).

The cytotoxic activity of resting NK cells and their precursors is rapidly potentiated by short exposure to IFN-α, β or γ (85). Studies using the single cell cytotoxicity assay have shown that virtually every stage in the interaction between NK cells and susceptible targets can be enhanced by IFN (16,86). When K562 or MOLT-4 cells are used as targets, IFN has the following potentiating effects: (i) the proportion of target-binding cells which are cytotoxic is increased; (ii) the time required for an NK cell to

lyse a bound target is reduced; (iii) increased amounts of natural killer cell cytotoxic factor (NKCF) are secreted and (iv) NK cell recycling time is reduced. Against anchorage-dependent target cells, IFN will also recruit and activate NK cell precursors from the cells which do not bind target cells. Maximum stimulation of NK activity by IFN-α is achieved within 4 -6 h, whereas the effect of IFN-γ takes 12-18 h to complete (85). Few studies have defined a dose-response curve for the action of IFN on NK cells. For IFN-α it appears that doses greater than 500 U/ml produce no further stimulation and doses greater than 1000 U/ml may be inhibitory (87). The action of IFN on NK cells also results in increased lysis of freshly isolated leukaemic blasts and lymphoma cell lines which are relatively resistant to resting NK cells (8,88).

Activation of NK cells by IFN may be counterbalanced by protective effects on some target cells. Preincubation of target cells with IFN permits binding by NK cells but lysis is reduced because NKCF release is not triggered (89). Little is known about the mechanisms underlying the immunomodulatory properties of the IFNs, including their activation of NK cells (83). Inhibitors of mRNA and protein synthesis block the effect of IFN on NK cells (90). Increased levels of 2'-5' Oligo A synthetase measured in NK cells after IFN treatment also suggest that common biochemical pathways underly many of the actions of IFN (85). However there was no correlation between the antiviral and NK-stimulatory profiles of different species and subspecies of IFN (85).

The most important pharmacological inhibitors of spontaneous or

IFN-activated NK cell activity are the prostaglandins (PGs). The addition of physiological concentrations of PGA_1, PGA_2, PGE_1 or PGE_2 to NK assays results in a rapid, but reversible loss of 50-75% of cytotoxic activity (90-94). The suppression of NK activity by activated monocytes and macrophages can be explained by their secretion of PGs (95,96). In a comparative study of human macrophages obtained from normal organs, those from the lung were the most potent inhibitors of NK cells (96). Macrophages associated with human malignancies have similiar suppressive properties and may account for the low levels of NK activity found in tumours and tumour-draining lymph nodes (97,98). Additional amounts of PG in tumours may also be derived directly from the neoplastic cells (92). In this context, IFN may mediate an antineoplastic effect by protecting NK cells from PG-induced suppression (99). Other cells not related to macrophages are capable of inhibiting NK activity, either via direct cell contact or by the release of soluble suppressor factor(s) (SSF).

A mixed lymphocyte culture-generated Leu-7^+ FcμR^+ lymphocyte inhibits NK activity by blocking binding between the NK cell and its target, possibly by direct competition for the recognition receptor (100). Unstimulated peripheral blood lymphocytes depleted of monocytes and cultured under serum-free conditions release a SSF which is capable of inhibiting more than 50% of NK cell activity (101). The identity of the SSF is unknown, but its activity is unlikely to be accounted for by prostaglandins or any known cytokine.

Reports in the literature suggest that NK cells are influenced by a

diverse range of cytokines, inflammatory mediators and hormones in addition to IL-2, IFN and PG's. Interleukin 1 (IL-1) is not an effective NK stimulant by itself, but its presence potentiates the increase in NK activity induced by IFN and /or IL-2 (102,103). Tumour necrosis factor (TNF) is capable of stimulating NK activity alone but its effect is also synergistic with that of IL-2 (82). Both IL-1 and TNF may be able to enhance the effect of IL-2 by the upregulation of NK cell IL-2 receptors (81,82). The neutrophil myeloperoxidase enzyme system has important bactericidal, inflammatory and immunoregulatory properties mediated by hypohalite ions generated from hydrogen peroxide and halides (104). Without affecting cell viability, products of the myeloperoxidase system cause a marked, but reversible suppression of NK activity (105). In animal models of tumour metastasis, oestrogens selectively inhibit NK activity and lead to an enhanced susceptibility to metastasis (106). In pregnant women and neonates, the significant depression of NK cell lytic function may be related to elevated levels of oestrogen hormones (107).

Large numbers of patients with advanced malignancy have been treated with purified or recombinant preparations of IFN because of its antiproliferative effects on tumour cells and stimulation of NK cells in vitro (108,109). In general, the results have been disappointing with little effect noted on carcinomas and a response rate of only 20% achieved for tumours such as multiple myeloma or non-Hodgkin's lymphoma. However, hairy cell leukaemia appears uniquely sensitive to IFN-α with complete remissions achieved in 90% of cases (110). It is postulated that NK

activity may be important in controlling this leukaemia as low NK activity noted in most pretreatment cases is restored to normal levels after IFN therapy. Continuous intermittent therapy with IFN produces a peak in peripheral blood NK activity one to three weeks after commencement of therapy, followed by a gradual decline to normal levels (109). The NK cells from patients treated with IFN are not active against NK-resistant targets, as is the case with cells treated in vitro. The reason for the consistent decline in NK activity with continued administration of IFN is unknown, but the induction of increased numbers of circulating T supressor cells by IFN is one hypothesis.

Interleukin 2 given intravenously to patients with disseminated malignancy produces a partial remission of the tumour in 13% of cases (111). Despite the intermittent injection of megaunit doses of IL-2, a consistent increase in peripheral blood NK activity has not been noted (112). However, the circulating precursors of lymphokine-activated killer (LAK) cells were depleted, suggesting that LAK or NK cells may migrate to peripheral organs during therapy with IL-2. Direct evidence showing that murine NK cells can be recruited from the circulation into the liver and lung after treatment with NK-stimulating agents has been reported (113). The bioavailability of recombinant IL-2 is limited by its extremely short half life which has been attributed to a lack of glycosylation (113). Preliminary studies confirm the efficacy of IL-2 given by continuous infusion but the in vivo immunological effects of this treatment modification remain to be reported (114).

1.5 Mechanisms of natural killer cell cytotoxicity.

Accumulating evidence from a vast literature on the mechanisms of NK cell cytotoxicity indicates that the process is a complex sequence of programmed events culminating in target cell lysis. Analytical methods allowing protein purification and characterization, lymphocyte cloning, membrane receptor isolation, monoclonal antibody synthesis and pharmacological inhibition of cellular function have all contributed to a coherent, but as yet incomplete concept of NK cell-mediated cytotoxicity (115). Target cell lysis by NK cells best fits a "stimulus-secretion model" which can be dissected experimentally into the following stages: (i) target cell binding; (ii) triggering of activation; (iii) NK cell activation and release of cytotoxic molecules; (iv) target cell death; (v) recycling and further lysis. Elucidation of the cytotoxic mechanism has largely been due to the studies of Targan et al (115,116) which exploit the different ionic requirements of the various stages. Binding occurs in the presence of magnesium ion (Mg^{+2}) and will not proceed to activation until calcium ion (Ca^{+2}) is added as a pulse. Neither the triggering nor lysis phases require Ca^{+2}, so the cytotoxicity assay can be arrested at each step and studied using the methods described above.

The initial binding of NK and target cells to form a conjugate involves interaction between an NK cell recognition receptor (RR) and a target structure (TS). Cold target inhibition studies of freshly isolated

and cloned NK cells illustrate that there are different RR, some of which are specific for only a single target while others can mediate the binding and lysis of several different cell types (6). The reactivity of an NK cell population against a range of susceptible targets is not based upon a clonal distribution of single RR's among NK cells. Instead, individual NK cells express a variable profile of different RR 's which may overlap partially with the receptor complement of other NK cells (5). Characterization of the NK RR has not progressed beyond preliminary experiments which suggest that the RR is a glycoprotein or glycolipid (53,117). Treatment of NK cells with proteolytic enzymes or metabolic inhibitors of glycosylation markedly inhibited the degree of binding to NK-susceptible targets. Murine data suggest that expression of the RR may be genetically determined by loci linked to the major histocompatibility genes (118). In contrast to NK cells, the RR on K cells mediating ADCC has been well characterized by phenotypic and functional studies as an Fc receptor for IgG (119). Once specific recognition is mediated by this receptor, lysis of the antibody-coated target proceeds by mechanisms identical to those of NK activity.

Target cell structures (TS) isolated from K562 cell membranes have been characterized as glycoproteins of molecular weight 200, 120 and 80 kD which are capable of selectively inhibiting the binding of NK cells to K562 cells (120,121). The two smaller molecules were also isolated from additional NK cell-susceptible but not resistant cell lines and demonstrated similar inhibitory binding to NK cells. Inhibition of the NK

cell binding function by pretreatment with nonphosphorylated sugars suggests that the TS may be glycosylated (122). The heterogeneity of TS was demonstrated in studies where the target specificities of NK cell populations were examined after adsorption of NK cell subsets on target cell monolayers (32). These data suggest that some TS may be common to all target cell lines whereas others are restricted in their distribution.

Candidate molecules postulated for the TS on human cells include the transferrin receptor, virus-induced glycoproteins and differentiation antigens. Data concerning the transferrin receptor are contradictory and apply only to some target cell types in the human NK cell system (123-128). The observed correlation between NK-susceptibility and transferrin receptor expression on nonmalignant or tumour targets and the sensitivity of NK activity to antitransferrin antibodies favour a role for this structure. The ability of malignant cell lines to compete with K562 cells for NK cell lysis also loosely correlates with their level of transferrin receptor expression, but complete inhibition of K562 lysis was not achieved with any of the lines tested (124). These studies imply that the transferrin receptor does not function as a complete TS, although it may act as an accessory NK-binding molecule for some, but not all target cells. Viral infection of target cells results in the expression of glycoproteins that are recognized by NK cells. In the case of Herpes simplex virus type 1 (HSV-1), the NK receptor is of viral origin and the degree of target lysis is directly related to the number of glycoprotein subunits expressed in the receptor (129). Surveys of nonmalignant cell

populations and tumour cell lines have highlighted the direct correlation between level of differentiation and susceptibility to NK cell activity (130,131). This relationship suggested that the TS may be a primitive developmental antigen. Supporting evidence came from a study where monoclonal antibodies to stage-specific embryonal antigen protected cell lines against NK-mediated lysis (131). Alternatively, the resistance of differentiated cells to NK activity may be explained by increased amounts of membrane sialic acid which shields the TS by steric hindrance (132).

The ability of NK cells to lyse cells devoid of detectable HLA antigens suggests that these structures are not prime targets for NK cells, but it does not preclude the possibility that HLA antigens associate with and influence the expression of TS on some cells (133). Evidence for this proposal comes from studies where the NK susceptibility of cell lines varied inversely with the levels of HLA class I antigen and β_2 microglobulin expression. Only one description of a single cloned NK cell line suggests that HLA antigens may also associate with the RR on effector cells (134). Antibodies against both class I and II antigens either enhanced or inhibited NK activity of this clone but the lack of effect on bulk NK cell populations suggests that the participation of HLA antigens in target cell recognition is a rare event.

Conjugate formation between NK and target cells occurs within minutes at temperatures between $4^{o}C$ and $37^{o}C$ and requires the presence of Mg^{+2}, but not Ca^{+2} ions (115,116). Inhibition of binding in a dose-dependent and reversible fashion by pretreatment with dimethyl

sulfoxide (DMSO) and 2-mercaptoethanol (2-ME) suggests that membrane protein-lipid structures and disulphide bonding participate in conjugate formation. The resistance of pre-formed conjugates to DMSO and 2-ME suggests however, that multiple binding mechanisms may maintain effective NK cell-target interaction.

Analysis of the cells binding NK-susceptible targets in a single cell cytotoxicity assay shows that a significant proportion may not be NK cells but are mainly T cells (16,135). These binding non-NK cells do not, however, make a significant contribution to the overall lysis of target cells. In similar assays, NK cells are also observed binding to NK-resistant targets. The membrane structures accounting for this nonspecific binding are unknown, although the family of leucocyte function antigens (LFA) are likely candidates (58). Blockade of the LFA-1 and LFA-2 (CD2) molecules on NK cells results in almost total inhibition of NK activity due to inhibition of conjugate formation (58). Furthermore, congenital deficiencies of LFA-1 are also characterized by deficient NK activity (136). The relative contributions of specific receptor interaction and nonspecific adhesion to effective NK-target cell binding are unknown. It is likely that both specific receptor interactions and indiscriminate binding mechanisms are required to produce maximum target lysis by NK cells.

Recent experiments using a panel of monoclonal antibodies directed against different epitopes on the CD2 and CD16 antigens on NK cells suggest that parts of both of these structures may be involved in NK cell

activation (137). Whereas the majority of antibodies studied inhibited NK cell activity, one directed against CD2 and another specific for CD16 stimulated cytotoxicity after binding. Thus the CD2 and CD16 structures may serve the dual functions of target cell binding and NK cell activation. The precise role of the CD2 and CD16 activation structures in the process of NK cell-mediated lysis remains to be defined.

Studies using the monoclonal antibody 13.1 have revealed a short triggering phase linking NK cell binding and activation. The 13.1 antibody is directed against an epitope carried by the polymorphic T200 glycoprotein which is found on the membranes of all PBMC including NK cells and cytotoxic T cells (138,139). After treatment with 13.1, cytotoxicity mediated by NK but not T cells was abrogated. Inhibition of NK activity towards K562 cells occurred only if the antibody was added before commencement of activation by Ca^{+2} pulsing. As no inhibition of NK binding was noted, 13.1 appears to be a potentially useful marker specific for the Ca^{+2}-independent triggering event. Experiments using inhibitors of transmethylation have confirmed the existence of a trigger stage. Transmethylation of phosphatidylethanoleamine in the cell membrane is important in cell activation and in increasing membrane fluidity (140). Inhibitors of transmethylation inhibit NK activity and appear to selectively block the triggering event, preventing progression to activation despite Ca^{+2}-pulsing (141,142).

Conjugate formation and triggering of the NK cell leads on to the "activation" or "programming for lysis" phase. During this period the NK

cell must remain bound to its target in order to deliver what is popularly known as the "lethal hit" (115,116). Morphologically this phase is characterised by the polarization of NK cell granules and secretion of granule contents in the vicinity of the bound target. Target cell lysis is able to proceed without NK cell contact once the "killer cell independant lysis" step (KCIL) begins. In order to determine the kinetics of the activation and KCIL steps, NK-target cell conjugates formed in the presence of Mg^{+2} alone are then pulsed with Ca^{+2} which is necessary for activation to proceed (115). At intervals after activation, the effect of conjugate disruption on target cell lysis is determined. These experiments have delineated an activation phase which may last up to 2 h for some effectors before maximum lysis of K562 or Molt-4 targets can be achieved. Addition of ethylenediaminetetraacetic acid (EDTA) at intervals after the calcium pulse has shown that the activation phase requires Ca^{+2} for only the first 90 min and thereafter the reaction can proceed independently. It must be appreciated that the duration of activation determined by calcium pulse experiments is the time taken for the entire population of NK cells to complete this phase. Kinetic studies of cytotoxic reactions show that detectable lysis of targets by some NK cells begins within minutes of conjugate formation. Unlike the binding stage, activation occurs optimally in a narrow temperature range around $37^{o}C$ (115).

Heterologous and monoclonal antisera which are known to inhibit NK cell activity have been used to probe for membrane structures crucial to

target cell lysis (143). The heterologous antiserum RH2 and monoclonal antibodies RH 7.2 and RH 17.2 were raised against sodium periodate-modified mixed lymphocyte culture-activated cytotoxic lymphocytes but the epitope(s) detected by these antibodies have not been identified. All three antibodies blocked NK cytotoxic activity at both the activation and KCIL stages and before the assay were shown not to react with the target cells. Therefore, blocking of activation was at the level of the NK cell whereas inhibition of KCIL was an effect on the target cell, implying that transfer of membrane components from NK to target cell was important for cytolysis.

Although the stages of NK cell-induced lysis can also be defined and subdivided using pharmacological agents, little is known about the site of action of these agents or how they produce their effect. The early, Ca^{+2}-dependent period of activation is blocked by inhibitors of Ca^{+2} flux into cells such as Verapamil and Lignocaine (116). The Ca^{+2} ion may be involved with the calcium-binding enzyme Calmodulin in regulating numerous enzymatic activities and indeed, the presence of Ca^{+2} in the cytosol is a known cofactor for granule secretion (116,144). Elevation of the intracellular cAMP/cGMP ratio by Prostaglandin E (PGE), theophylline or cAMP also inhibit NK activation (116,145,146). Both PGE and 2-ME block the late, Ca^{+2}-independant phase of activation as well as KCIL (116). In the activation stage, PGE inhibits NK cells by increasing intracellular cAMP but interruption of KCIL is attributed to a cytoprotective effect of PGE (116).

The participation of free oxygen radicals in NK cytotoxicity is suspected because chemically unrelated scavengers of the hydroxyl radical ($OH^{\cdot}$) are known to abolish NK activity without affecting the frequency or viability of effector-target conjugates (147,148,149). It has been suggested that peroxidation of NK cell lysosomal membranes by $OH^{\cdot}$ facilitates secretion of cytotoxins in a fashion similar to that proven for mast cell granules (150). There is mounting evidence however, against free radical production by NK cells (151). Although free radical production is detected by chemiluminescence seconds after contact between NK cells and susceptible targets, it is likely that the actual producers are a minority population of monocytes contaminating enriched LGL preparations. Further evidence against NK cell $OH^{\cdot}$ production is the intact NK activity found in chronic granulomatous disease which is characterized by the absence of free oxygen radical generation due to genetic defects in the NADPH oxidase system (152). However NK cells may produce small amounts of $OH^{\cdot}$ as byproducts of the lipoxygenase pathway (153). This hypothesis is supported by the inhibitory effects of lipoxygenase inhibitors on NK activity (148) while potent inhibitors of $OH^{\cdot}$ production by the NADPH oxidase pathway are without effect (148,149). The issue of $OH^{\cdot}$ function in NK activity remains to be resolved. It is possible that NK cells may generate small amounts of $OH^{\cdot}$ in cellular microenviroments which are undetectable by present methods.

The activation of NK cells is signalled by morphological changes affecting the LGL secretory apparatus. Within minutes of binding to a

sensitive target, the Golgi apparatus and microtubule organizing centre of the NK cell polarizes towards the area of mutual cytoplasmic contact. Granules derived from the Golgi are then guided by microtubules to the vicinity of membrane contact (154). Whether granule contents are transferred onto the target surface directly from the effector's membrane or via secretion into the extracellular space is unresolved. Natural killer cells bind to targets by finger-like protrusions which intrude into reciprocal pockets in the target cell as well as by broad areas of intimate contact (154). Interference with the secretory process of LGL shows that release of the granules is vital to delivery of the lethal hit. Disruption of vesicular traffic from the Golgi to the cell surface by microtubule inhibitors inhibits NK activity and chemicals which degranulate lysosomes or pervert their function have the same effect (155,156). Genetic defects of lysosomes reported in the beige mouse or in man (Chediak-Higashi syndrome) are also characterized by absent NK activity (157,158).

After delivery of the lethal hit, target cell destruction occurs whether or not the NK cell remains attached. The progression to lysis is not inevitable as evidenced by the protective effect of PGE and of modulators which increase the rigidity of lipid membranes (116). There is some controversy surrounding the processes involved in NK cell-mediated target cell lysis. The formation of membrane pores has been observed in target cells after attack by CTL, the membrane attack sequence of complement and NK cells (159,160). However, careful ultrastructural studies question the role of pore formation and suggest that target cells

may die by apoptosis. In this distinctive process, the target cell cytoplasm and nucleus are actively segregated into multiple membrane-bound vesicles which are endocytosed by adjoining parenchymal and scavenger cells (161). The nature of the lytic molecules mediating apoptosis is unknown and the overall relative importance of the multiple lytic molecules secreted by NK cells remains to be determined.

Cytolysins so far associated with NK cells include perforin, NK cell-associated cytotoxic factor (NKCF) and a collection of proteases and esterases (162,163). All are granule-associated and are released during the activation stage of NK cytolysis. Perforin is probably the most significant cytotoxin secreted by NK cells (162). Most is known about this molecule because of its role in killing by cytotoxic T lymphocytes (CTL) and its similiarity to the ninth component of complement (C9). Polymerization of the 60-75kD perforin molecule in the presence of Ca^{+2} results in the formation of a cylindrical hollow channel which is able to insert into the lipid bilayer of the target cell membrane (160). The resulting pore is postulated to allow free inflow of electrolytes and other solutes into the cell, leading to osmotic lysis. Large pores comprised of polyperforin (internal diameter 16 nm) and smaller structures (6 nm) thought to be single perforin molecules, have been visualized by electronmicroscopy in the membranes of cells after lysis by complement, NK cells or CTL. Perforin is antigenically similiar to C9 and considerable homology exists between their genes, making it likely that the perforin gene arose from the C9 gene by a primitive reduplication event. The

granular location of this molecule is suggested by the formation of identical membrane pores by isolated and purified NK cell granules. Purified perforin or isolated NK granules possess potent nonspecific cytotoxic properties extending well beyond NK-sensitive cells to include most nucleated cell lines (159,162). It is postulated therefore, that the specifity of lysis by NK cells is conferred by prior binding and activation steps. Bystander damage to cells near NK reactions may be avoided by the intimate nature of the contact between target and effector which prevents leakage of perforin into the extracellular space. Damage to the NK cell itself may be limited by granule derived sulphated proteoglycans which can bind to and neutralize excess liberated perforin (164).

The existence of NK cell-derived cytotoxic factor (NKCF) was first suspected when the supernatants from cocultures of NK cells and their susceptible targets were found to have cytotoxic activity (165,166). Unlike perforin, NKCF retains the target specificity profile of the secretor NK cell population. This property implies the existence of specific receptors on target cells which are recognized by NKCF molecules. The nature of the NKCF-receptor system remains elusive. Inhibition experiments with phosphorylated sugars show that this receptor system is independent of membrane structures which mediate initial target recognition and binding (167). It is also evident that the release of NKCF is relatively nonspecific as NK-resistant tumour cell lines can bind to and stimulate NK cells to release NKCF. The specificity of NKCF is therefore at the level of the target cell which will only be lysed if receptors

specific for NKCF are expressed. The target cell receptors for NKCF are poorly understood but appear to contain protein because of their sensitivity to papain (168). Natural killer cytotoxic factor is a 20-30,000 kD MW glycoprotein which appears to be located in NK cell granules because of its inhibition by antigranule antibodies (168,169). Both increased cytosol Ca^{+2} and protein kinase C are involved in the release of NKCF. In patients with AIDS, defective release of NKCF underlies the defective NK activity seen in this infection (170). A paradox evident in NKCF studies was that the NKCF produced by NK cell-target cocultures was only weakly cytotoxic unless incubated in concentrated form with targets for periods of up to 40 h (168). However NKCF may function as a significant cytolysin when released into the microenviroment of the NK-target cell contact area. The mechanism or site of action of NKCF is unknown but recent studies have convincingly demonstrated that NKCF activity was not attributable to, or enhanced by, tumor necrosis factor (TNF), Lymphotoxin (LT) or IFN (169).

Some NK cell secretory products have an antineoplastic action by inhibiting tumour cell proliferation rather than directly causing lysis. The IFNs secreted by NK cells are prime candidates for this cytostatic role (83). A 44kD protein synthesized by NK cells has been called Leukoregulin because it also has antimitotic effects against NK cell-susceptible tumour cell lines (171). In addition to its cytotoxic properties on some tumour cells, TNF is also cytostatic (172,173). This cytokine is released by either resting or IFN-γ activated NK cells but does not appear to contribute

significantly to the cytostatic or cytotoxic effect on NK cell-susceptible targets in vitro (168,172). However under special conditions in vivo, NK cell derived TNF may have a significant effect.

The third group of toxic molecules involved in the cytotoxicity of NK cells are serine proteases, serine esterases, arylsulphatase, phospholipase and possibly endonuclease enzymes located in the NK cell granules (162,174-176). Current evidence suggests that the serine proteases are instrumental in enzymatically activating NKCF or perforin in an enzymatic cascade similiar to that of the complement system. There may be a limited role for phospholipase and arylsulphatase in mediating direct injury to cell membranes and the degradation of nuclear DNA early in cell lysis may be mediated by passage of enzymes through membrane pores to the nucleus.

Mathematical analysis of the data from single cell and ^{51}Cr-release cytotoxicity assays run simultaneously suggests that a single NK effector is capable of lysing several target cells in succession (177). During a four h cytotoxicity assay, the average number of K562 or MOLT-4 targets killed by a single LGL is estimated to be 3.2 and 4.5 respectively. This recycling capacity of NK cells implies that lysis involves a tightly controlled secretory process which allows sufficient reserves for further target cell encounters in a brief period of time. The relatively slow regeneration of granule contents after degranulation suggests that only a fraction of the NK cell's cytolysins are used during each lytic encounter (168).

In summary, the complicated sequence of steps necessary for target

lysis by NK cells is best explained by three receptor-mediated events which all contribute to the overall specificity of the effector-target interaction. The first set of receptors mediates effector-target binding and is followed by NK cell activation which is mediated by a second set. Finally, NKCF must interact with a third specific receptor on the target cell surface before lysis is effected. There is evidence to suggest partial sharing of antigens or membrane structures between the receptors mediating this complex sequence. The participation of the same membrane glycoprotein from the K562 cell line in both effector cell binding and lysis by NKCF suggests that the receptors for these events may share membrane structures (178). Antigenic similiarities between receptors are further suggested by the inhibition of both binding and activation stages by a single antibody specific for this glycoprotein.

The enigmatic receptor system responsible for the cytotoxic activity of classical $CD3^-$ NK cells is different from that used by a small population of $CD3^+$ peripheral blood lymphocytes which also mediate spontaneous NK activity (39). It has been shown that the T cell receptor and adjacent CD3 activation complex may mediate the nonspecific cytotoxic activity of these T cells. The target cell structure specifically recognized by this receptor is a 140 kD glycoprotein heterodimer which is an activation marker on a wide range of cells (179). Despite its wide distribution, this molecule is not recognized by NK cells. Like antigen-specific reactions, the nonspecific cytotoxicity of $CD3^+$ NK cells can be blocked by monoclonal antibodies against either the T cell receptor

or the CD3 antigen (39). This receptor system is not available to $CD3^-$ NK cells which do not rearrange the genes encoding for the T cell receptor.

1.6 The antineoplastic role of natural killer cells.

Abundant evidence exists to support an important role for NK cells among the defence mechanisms which prevent the development and spread of malignancy. The most convincing data come from experimental models of malignancy in rodents where the background NK activity, timing, site of tumour induction and NK-susceptibility of the tumour can be manipulated independently. These investigations form a basis for the discussion of clinical studies of patients with malignancy where the evidence for an antineoplastic function for NK cells is, by its nature, largely circumstantial.

In rodent models of malignancy, NK cells are effective against a wide range of leukaemias, lymphomas, carcinomas and sarcomas (180-187). These tumours were induced in vivo by inoculation of established tumour cell lines or by chemical carcinogens or irradiation. In these studies, there was a clearcut inverse correlation between the level of pre-existing splenic and peripheral blood NK activity and the subsequent development of malignancy (181,182). Natural killer cell activity was diminished in vivo by the use of immature animals, genetic variants with low NK activity (the beige mouse) or by the chemical suppression of NK activity using agents such as β-oestradiol (185). The supplementation of

NK activity by adoptive transfer of functional cells or treatment with IFN was then followed by restoration of resistance to the development of malignancy (181,182). This effect however, was noted only with small starting doses of tumour and furthermore, once the malignancy was well-established, the level of NK activity of the host was found to be irrelevant to further progression (184). The selective effect of NK cells on tumours in vivo was studied against NK cell-susceptible or resistant mutants of the same tumour cell line which had identical susceptibility to lysis by activated macrophages and cytotoxic T lymphocytes (186). These experiments showed a direct correlation between the tumourigenicity of the cell line mutants and their resistance to NK activity. Natural killer cells were also effective in reducing the incidence of lung metastases complicating primary footpad tumours or implanted following intravenous innoculation of tumour cells (185-187). Reconstitution experiments suggest that NK cells exert maximal suppression on metastasis formation within 24 h of tumour cell entry into the circulation. After this time, numbers of malignant cells sufficient to produce metastases may have entered tissues where they were inaccessible to NK activity (187). Conclusions drawn from animal models where tumours are experimentally induced may not apply to tumours arising spontaneously in humans. Because of the constraints of time, experimental animals are rarely observed for long enough periods to relate the incidence of spontaneous malignancy to the level of NK cell activity. Nevertheless, one such study of beige mice has documented a twofold increase in spontaneous tumours

in homozygous as compared to heterozygous littermates (188).

Due to the nonspecific suppressive effects of large tumour burdens and cancer therapy, a definitive role for NK cells in human malignancy has been more difficult to prove (189,190). Peripheral blood NK activity against K562 targets is preserved in the early stages of most malignancies but NK activity declines as the disease progresses (189). Lymphomas and leukaemias are exceptions to this observation as NK activity is significantly impaired even at the earliest stages of these tumours prior to therapy (191,192). The reduced NK activity in advanced malignancy has been attributed to hyporesponsiveness to IFN, decreased recycling capacity and to reduced numbers of circulating Leu-7^+ NK cells (193-195). In acute and chronic leukaemias, defects in NK cell binding, lytic activity and recycling time all contribute to low levels of NK activity (192). A similiar early suppression of NK activity may exist in malignant melanoma where NK activity against a melanoma cell line target was enhanced after surgical excision of early lesions (196). Thus with the possible exception of select tumours, pre-existing defects in peripheral blood NK activity do not appear to predispose to human malignancy.

A more fundamental analysis of the role of NK cells in human maligancy can be made by using freshly isolated tumour cells as targets to assess the cytotoxic potential of lymphoid cells isolated not only from the peripheral blood, but also from organs affected by malignancy and the tumour itself. Such organs frequently affected by malignancy have variable levels of endogenous NK activity. The intestinal lamina propria

displays minimal levels of NK activity against either K562 cells or freshly isolated colon carcinoma cells (197). On the other hand, cells from the lung interstitium and alveolar spaces mediate significant NK activity against K562 cells (26,198). Testing peripheral blood lymphocytes (PBL) and tumour-infiltrating lymphocytes (TIL) for cytotoxic activity against freshly isolated tumour cells yields conflicting results. While a number of investigators report no spontaneous PBL cytotoxicity against fresh targets (11,199), there are other reports of significant levels of tumour-directed PBL cytotoxic activity in up to 50% of normal donors and patients with malignancy (12,13,199,200,201). Immunohistochemical studies suggest that NK cells are infrequent in malignant tumours (202), but functional studies of isolated TIL show low grade but significant activity against K562 cells and fresh tumour cells in 10-50% of cases (203-205). Tumour cells isolated from breast, colon and lung carcinomas demonstrated susceptibility to lysis by both PBL and TIL. Positive identification of the anti-tumour effector cell(s) in PBL and TIL populations has not been achieved. However in some studies an IFN-inducible large granular lymphocyte was isolated with cytotoxic activities against both autologous and allogeneic targets (23,200,203,205). Although the evidence therefore favours an NK cell, some studies indicate that cytotoxic T cells may also be involved in antitumour cytotoxic activity (206).

Epidemiological studies of apparently normal individuals at increased risk for malignancy support a role for NK cells in tumour immunesurveillance. There is a tendency for the relatives of patients

with solid tumours to have significantly reduced peripheral blood NK activity (9,207). In the case of malignant melanoma, significant depression of NK activity was detected only against melanoma cell line targets. Furthermore, the level of NK activity in normal subjects exposed to carcinogens such as ultraviolet light or tobacco smoke is significantly lower than unexposed controls (208,209). The increased rate of maligancy in immunodeficiencies such as Chediak-Higashi disease, severe combined immunodeficiency and the acquired immunodeficiency syndrome has been attributed to reduced or absent NK activity (42,210,211), but in these disorders, defective NK activity is usually accompanied by numerous other immune abnormalities which could equally well predispose to neoplasia.

The general conclusions from animal models of malignancy is that NK cells are primarily effective in preventing the emergence of malignant cell clones. A secondary role for NK cells may lie in their ability to rapidly neutralize haematogenous metastases. It is difficult to define such a clear role for NK cells in human malignancy. Against selected tumours such as lymphoma, NK cells may be an important primary defence and in unusual cases of familial malignancy, a defect in NK activity may lower resistance to malignant transformation. The presence of small numbers of tumour-reactive NK cells in the peripheral blood and tumours of some individuals further suggests an active role for NK cells in human malignancy. On balance, NK cells are likely to be important as a surveillance mechanism early in tumour development in humans rather than a significant influence after the tumour has presented clinically.

1.7 The antimicrobial properties of natural killer cells.

There is abundant evidence available to suggest that NK cells form an intergral component of host defence against certain viruses, bacteria, fungi and parasites. The bulk of these data is circumstantial and depends upon demonstration of a correlation between resistance to infection and level of NK activity or upon the ability of NK cells to lyse microbial pathogens in vitro. In addition, most studies utilize murine NK cells which are known to differ biologically from human NK cells, particularly in organs such as the intestine (212). Based on evidence of this nature, NK and K cells have been implicated in resistance against a wide range of bacterial, fungal and protozoan pathogens including Salmonella (213), Shigella (47,214), Legionella (215), Cryptococcus (49,216), Giardia (48) and Trypanosoma (217).

Much more is known about the interaction between NK cells and viruses. After viruses penetrate the host's physical defences, readily inducible (eg IFN activated) NK activity may limit viral dissemination until specific responses by T and B cells evolve. This role for NK cells was first suggested by the prominent proliferation and activation of NK cells noted early in some viral infections (218) and was later confirmed in models of murine cytomegalovirus (CMV) infection (219). Identification of NK cells as the mediators of early resistance to viral infection depended on the following clinical and experimental observations: (i) cytotoxic activity against virus-infected cells was comparable in subjects with or

without serological evidence of prior infection; (ii) resistance to infection in vivo correlated closely with the level of NK activity against virus-infected cells in vitro; (iii) addition of NK cells to infected cell monolayers limited viral replication; (iv) murine adoptive transfer and in vivo depletion experiments identified the anti-virus effector cells as NK cells; (v) effector cells in human studies were $CD16^+$ $OKM1^+$ $Leu\text{-}7^+$ Ia^- LGL and (vi) cytotoxicity against virus infected targets was not HLA-restricted and was rapidly boosted by IFN-α or β and IL-2 (220-226). In some viral infections such as murine lymphocytic choriomeningitis, elevated NK activity may be an epiphenomenon that has no bearing on the progression of infection (227,228).

The rapid increases in NK activity and IFN levels in early viral infections act synergistically to limit viral replication. In murine CMV infections, high levels of circulating IFN-α have little protective effect without an intact NK system (219). The main function of IFN-α in CMV infection appears therefore, to be the augmentation of endogenous antiviral NK activity rather than a direct antiviral effect. Investigation of human CMV infections with IFN-neutralizing antibodies in vitro indicates that production of IFN-α was mandatory for anti-CMV activity (221,226). Characterization of the IFN-secreting cell showed that an Ia^+ $CD16^-$ $OKM1^-Leu\text{-}7^-$ mononuclear cell without NK activity was the source (221). The lack of correlation between the levels of HSV-1 induced IFN-α production and NK activity in patients with the acquired immunodeficiency syndrome also suggests that NK cells are not an important source of IFN-α

(228). As well as increasing the cytotoxicity and target-binding capacity of antiviral NK cells, IFN may also modify the susceptibility of target cells to lysis. Uninfected target cells such as fibroblasts may have reduced, equivalent or increased sensitivity to NK activity when compared with infected targets (230). In the case of human and murine CMV, treatment with IFN reduces the sensitivity of uninfected targets to lysis but leaves the sensitivity of infected cells unchanged (231,232). This effect may selectively protect uninfected host cells from NK cell-mediated cytotoxicity. Similar experiments using viruses resistant to NK activity show that IFN markedly reduces the lysis of both infected and uninfected target cells (231).

The nature of the membrane structure on virus-infected target cells mediating recognition and lysis by NK cells is obscure and evidence suggests that the target structure may be either a host cell or a viral product. On the one hand, competition between CMV or HSV-1 infected and uninfected fibroblasts in cytotoxicity assays suggests that the NK cell receptor is also found on uninfected cells and may therefore be a modified or enhanced component of the normal cell surface (231,232). However under different experimental conditions, NK cells appear to lyse HSV-1 infected cells only if a membrane glycoprotein of viral origin is expressed (130). The NK cell receptor on CMV-infected fibroblasts is expressed early in the course of infection, coinciding with the virus induced synthesis of "early proteins" which precedes assembly and release of intact virions by up to 40 h (130,232,233). This property of NK cells may

limit CMV infection at an early stage before large numbers of complete virions are released from infected cells. The target cell structure recognized by NK cells may be expressed independently of major antigenic variations in viral products which are also expressed on the infected cell's surface. Whereas specific T cell responses to different strains of the influenza virus must evolve to match antigenic shifts in haemagglutinin and neuraminidase determinants, NK cells from the same donor are active against different strains of virus irrespective of their antigenic variability (234).

Subsets of NK cells mediating cytotoxicity exclusively against viruses or tumour targets can be identified. Cold target inhibition studies show that NK cells with activity against HSV-1 overlap minimally with those mediating cytotoxicity against K562 cells (31,230). While both subsets of cells were $CD16^+$ $Leu\text{-}7^+$ $OKM1^+$, anti-K562 effectors were $CD2^+$ Ia^- whereas those active against HSV-1 were $CD2^-$ Ia^+. Further evidence supporting the independence of NK cells active against K562 and HSV-1 infected fibroblasts is the dissociation found between the levels of these activities in some normal human subjects (31). The apparent differences between antiviral and antitumour NK cells may however depend more upon the type of target cell used to carry the virus rather than the nature of the infecting virus itself. When, for example, HSV-infected lymphoblastoid cell lines are used as targets there is no detectable difference between the antiviral NK cells and those which lyse K562 cells (235).

Spontaneous and IFN-augmented human NK cell activity has been detected against other important viral pathogens including the Epstein-Barr virus, Measles, Mumps, Vaccinia, Influenza, Varicella, Human T Cell Lymphotrophic Virus type 1 and the HIV-1 (230,234,236-238). The demonstration that NK cells can lyse targets infected with these viruses in vitro offers only circumstantial evidence that NK cells are important in influencing the course of these infectious diseases. Studies probing for an immunoregulatory role of NK cells in resistance to infection are infrequent, but they illustrate that the anti-viral effect of NK cells may act through mechanisms other than direct cytotoxicity. In the case of influenza, NK cells provide accessory cell function for the generation of specific cytotoxic T cells in addition to mediating nonspecific cytotoxicity (239).

1.8 Regulatory functions of natural killer cells.

In addition to their nonspecific cytotoxicity against tumours, cells belonging to the NK lineage contribute to the regulation of nonmalignant cellular growth and differentiation. Following an early report that immature thymocytes were susceptible to lysis by NK cells, other populations of rapidly proliferating precursor cells in the bone marrow and peripheral lymphoid tissue were examined for the presence of NK cell-mediated control mechanisms (240-242). Lymphoid and haemopoietic cells from the thymus, spleen and bone marrow were susceptible to NK

activity with those cells in earlier stages of differentiation or from foetal tissue showing the greatest sensitivity to lysis. Consistent with NK cell-mediated cytotoxicity were the comparable sensitivies of autologous or allogeneic targets to lysis and their ability to compete effectively with NK cell-susceptible tumour cell targets in cytotoxicity assays. An expanding body of evidence now indicates an important regulatory role for NK cells mediated through both their cytotoxic capacity and their secretion of cytokines and proinflammatory mediators.

Stem cell growth and differentiation in the bone marrow microenviroment is regulated by the colony stimulating factors (CSF's) and bone marrow stromal cells (243,244). Additional control of stem cell proliferation may be mediated by NK cells either by direct lysis or by the secretion of colony inhibiting or stimulating factors. Numerous studies show that preincubation of bone marrow stem cells with NK cells retards the subsequent development of differentiated erythroid, granulocytic/monocytic and mixed (erythroid/granulocytic/monocytic/ megakaryocytic) colonies in semisolid agar culture or in vivo (245-250). An identical inhibitory effect was achieved by pretreatment of bone marrow cultures with the soluble products of NK cells which had been activated by brief contact with either nonadherent Ia^+ bone marrow cells or NK cell-susceptible tumour cells (249). Although the profile of bone marrow colony types affected varied according to the purity of isolated NK cell preparations, colonies arising from more primitive precursor cells were more consistently inhibited. The nature of the interaction between

NK cells and haemopoietic stem cells is unclear, but there is convincing evidence to suggest that bone marrow cells may be lysed directly by NK cells after an initial contact phase (251). Mediators secreted by NK cells may also exert a significant cytostatic effect on bone marrow cells by inhibiting their proliferation in response to the CSF's, but the colony inhibiting activity secreted by NK cells following contact with bone marrow cells (NKCIA) has not been characterized extensively (249). There is some physiochemical evidence indicating that NKCIA is identical to NKCF which mediates the cytotoxic effect of NK cells against tumours (252). Additional studies suggest that NKCIA is a heterogeneous collection of marrow inhibitory factors which may also contain IFN-γ and TNF (246,252,253). Inhibitory activity against bone marrow cells is restricted to a population of large granular lymphocytes with the CD2, CD16 and Leu-7 antigens typical of NK cells (250). Despite the sparsity of NK cells in human bone marrow, regulatory cellular interactions are nevertheless feasible with small numbers of cells (40). However not all interactions between NK cells and haempoietic stem cells may be inhibitory. The reported promotion of peripheral blood stem cell erythropoiesis by LGL may be attributable to their secretion of colony stimulating factors (254,255). The physiological significance of this observation is uncertain however, as the effect of NK cells on haemopoiesis in vitro is almost always inhibitory.

The recognition that NK cells regulated haemopoiesis stimulated interest in the role of NK activity in bone marrow transplantation. As a

result, NK cells have been implicated in the pathogenesis of graft rejection and graft versus host disease (GVHD). In the murine model of bone marrow transfer from identical inbred parents to irradiated F1 hybrids, graft rejection occurs by an unknown mechanism independent of major HLA antigens and T cell mediated immune responses (256). Adoptive transfer and cell depletion experiments in vivo suggest that NK cells mediate this hybrid resistance (183,257), but at present there is no direct evidence for a similar mechanism of graft rejection in humans. Circumstantial evidence linking NK cells to GVHD in humans arose from striking correlations discovered between the incidence of GVHD and high levels of pre or post transplant NK activity (258,259). More direct support came from a model of murine GVHD where in vivo depletion of recipient NK cells alleviated the disease (260). These observations suggest that NK cells in the recipient may act as stimulators of GVHD. Alternatively the abnormalities of NK activity in GVHD may be secondary to the elevated levels of interferon documented after bone marrow transplantation (261). The ability of NK cells to serve as stimulator or accessory cells in mixed lymphocyte reactions provides a possible mechanism of action for these cells in graft rejection and GVHD (262,263).

Natural killer cells may intervene at several stages in the differentiation of the antigen-stimulated B cell to either inhibit or support the development of a specific antibody response. In the bulk of studies which demonstrate NK cell-mediated suppression of spontaneous or pokeweed mitogen-induced antibody production, the mechanism of the

inhibition is unclear (264-270). Nevertheless, it is known that antigen-presenting cells, particularly after encounter with antigen, are susceptible to inhibition by NK cells (268,271). There is also suggestive evidence that NK cells are able to lyse B cells by contact initiated release of NKCF. In these experiments, NK cell-mediated suppression of antibody production by B cells was negated by competition with NK-sensitive K562 cells and by treatment of NK cells with a monoclonal antibody against the K562 target recognition structure (272). Natural killer cell derived IFN may also directly suppress antibody production and the presence of IFN also enhances the suppressive effects of NK cells on antibody production (269,273,274). Comparative studies with T suppressor cells suggest that NK cells are the more potent inhibitors of antibody production and in some assay systems, NK cells maintain effectiveness even when comprising less than 1% of the total cell population (263,264). Paradoxically, antibody production may also be supported by NK cells by virtue of their secretion of a B cell growth factor (BCGF) and IL-2, both of which stimulate the proliferation of B lymphoblasts (255,275,276). These functional observations are consistent with the presence of Leu-7^+ LGL in the germinal centres of lymphoid follicles (277,278) where NK or related cells would be strategically placed to influence B cell development. Less is known about the effect of NK cells on T cell growth and differentiation. The maturation of primitive T cells in the human thymus may be limited through NK-mediated lysis (242) and as producers of IL-2 and IFN , NK cells provide direct support for the proliferation and activation of

post-thymic T cells. Natural killer cells expressing the OKM1 and Ia antigens provide antigen-presenting or accessory cell function for T cell-mediated reactions to alloantigens or polyclonal stimulants (262,263). There are reports that NK cells inhibit mixed lymphocyte reactions but this effect was only noted after activation of the NK cells by immune complexes containing erythrocytes or by IFN (266,267).

Many of the regulatory functions of NK cells are based upon their capacity to secrete immunomodulatory cytokines. The release of IFN- α and IFN–γ, IL-1, IL-2 and CSF's from NK cells after mitogenic or microbial stimuli is well documented (254,273,275,279). Interleukin 2 is also a potent stimulus for IFN- γ secretion by NK cells (84). Other mediators released from NK cells relevant to the control and amplification of the inflammatory response include an uncharacterized chemotactic factor, TNF and plasminogen activator (173,280,281). On a per cell basis, the secretion of IL-1 and IL-2 exceeds that of the macrophage and T cell, implying that LGL are physiologically important producer cells (254,279,282). Phenotypic and functional studies suggest that the NK cell population responsible for cytokine secretion is heterogeneous and that partially overlapping subsets of cells may produce different cytokines. The phenotypic and functional characteristics of freshly isolated LGL producing cytokines is summarized in Table 1.2.

Studies of cloned NK cells confirm that they have potent secretory potential (5). Using these homogeneous populations, it was demonstrated that a single NK cell can produce more than one cytokine and that a

Table 1.2 The surface phenotypes of large granular lymphocytes capable of producing cytokines.

Cytokine secreted *	Phenotype@	Reference
IL-1	$CD2^{+/-}16^{+}$ $OKM1^{+}$ Ia^{+}	279
IL-2	$CD2^{+}3^{-}$ $OKM1^{+/-}$ Ia^{+} $Leu\text{-}7^{-}$	254,282
IFN-γ	$CD2^{+/-}3^{-}16^{+/-}$ $OKM1^{+/-}$	282,283
BCGF	$CD3^{-}16^{-}$ $Leu\text{-}7^{+}$ $OKM1^{+}$	275

* IL-1 (Interleukin 1); IL-2 (Interleukin 2); IFN-γ (gamma Interferon) and BCGF (B cell Growth Factor).

@ Presence of surface antigen indicated by +; absence by -; +/- indicates the surface antigen may be either present or absent.

secretory cell can be either cytotoxic or noncytotoxic.

1.9 Characteristics of lymphokine-activated killer (LAK) cells.

Lymphokine-activated killer (LAK) cells are a heterogeneous collection of activated lymphocytes distinguished by their ability to lyse tumour cells which are resistant to NK cell activity (11,27,197,284). Numerous tumour cell lines which are either resistant or sensitive to NK cells are lysed by LAK cells and furthermore, freshly isolated tumour cells are also highly susceptible to the cytotoxic activity of LAK cells. Malignant cells obtained from several types of carcinoma, melanoma, hepatoma, sarcoma, lymphoma and leukaemia are reportedly destroyed by LAK cells prepared from PBL (11,192,285-287). There is disagreement concerning the capacity of LAK cells to damage nonmalignant cells. Autologous and allogeneic PBL were reported to be either resistant or susceptible to highly active LAK cells (288-290). Nonlymphoid targets including endothelial and epithelial cells and fibroblasts were also found to be lysed by LAK cells in one study (291). Like the nonspecific cytotoxicity of NK cells, LAK cell activity is operative on first exposure to the target and is not MHC-restricted. Peripheral blood LAK cells from cancer patients or normal controls are equally effective against either allogeneic or autologous tumour cell preparations (11,27,285). Although resting NK cells may display low grade activity against fresh tumour cells, LAK cells generally have a much greater cytotoxic effect (11-13).

In contrast to the two-signal mechanism of T cell activation, only exposure to IL-2 is required for the generation of LAK cells (11,27,294). The inhibition of LAK cell generation by the presence of antibodies to the IL-2 receptor suggests that interaction of IL-2 with its receptor is the triggering event (27). Cellular proliferation is essential for the activation of LAK precursor cells, as inhibitors of cell division prevent the induction of LAK cell activity (11,197,295). The LAK activity of PBL exposed to optimal concentrations of IL-2 reaches maximum levels after 6-7 days in culture. Lymphocyte populations with LAK activity contain a high proportion of proliferating lymphoblasts with plentiful cytoplasm containing a variable number of small azurophilic granules (11,27,292). Although IFN-α or γ and IL-1 do not appear to participate in LAK cell generation, the involvement of other cytokines or cell types in intermediate steps has not been excluded (296). A recent report shows that murine splenic but not peripheral blood LAK cells can be generated by IL-4, either alone or in combination with IL-2 (297). It remains to be shown whether human LAK precursor cells can be activated by IL-4 . The induction of LAK cells is subject to inhibition by exposure early in culture to PHA-induced suppressor cells or activated macrophages (298,299). Paradoxically, the addition of peripheral blood monocytes to LAK precursor cells enhanced culture-generated LAK activity by an unknown mechanism (299). The process of LAK cell generation is unaffected by the presence of Cyclosporin, whereas hydrocortisone is inhibitory (197,295). As one of the prime actions of Cyclosporin is to inhibit antigen-induced IL-2

production by lymphocytes, this result shows that induced IL-2 production by cells in culture is not necessary for LAK cell generation. The mechanism accounting for the suppressive effect of hydrocortisone on LAK cell generation is unknown and further investigation of this pharmacological effect may reveal more about the LAK cell phenonemon (197,295).

Although Grimm et al first described the generation of LAK cells by exposing PBL to highly purified or recombinant IL-2, cytotoxic cells with similar properties are also detected after exposure of PBL to T cell mitogens (300), viruses (301) or alloantigens (302-308) in a mixed lymphocyte culture (MLC). Mixed cultures containing lymphocytes and autologous or allogeneic tumour cells (MLTC) lead to the formation of both tumour-specific cytotoxic T lymphocytes and LAK cells (308). It is probable therefore, that LAK cells will be generated during any immunological event which generates enough IL-2 to activate LAK precursor cells. Cells with LAK activity which are generated by methods other than direct exposure to IL-2 have been called NK-like cells (302,303), activated lymphocyte killer (ALK) cells (285), anomalous killer cells (298) and promiscuous killer cells (290). In future, all these effector cells should be collectively classified as LAK cells, because possible differences in induction requirements and phenotype do not justify separate designations.

Analysis of LAK cell surface markers reveals a heterogeneous array of effector cell phenotypes (Table 1.3) which vary according to the

method of generation and precursor cell population used. In MLC, MLTC or after mitogen exposure, LAK cells are predominantly T cell-derived and express T cell phenotypes (299,302-304,307,308). When PBL are exposed to IL-2 in culture, LAK cells with the phenotype of NK cells predominate. These $CD2^{+}3^{-}16^{+}$ Leu-$7^{+/-}$ Leu-19^{+} LAK cells are the most frequent, because of lower IL-2 requirements and shorter generation times compared to the other types of LAK cells (38,60,309). Experiments using sorting procedures to prepare pure populations of precursor cells show that LAK cells with NK cell phenotypes are derived from resting NK cells. A minority population of LAK cells generated in an MLC are derived from peripheral blood NK cells and are $CD3^{-}16^{+}$Leu-7^{-} (305,306). These LAK cells derived from NK cells were previously described by some authors as NK-like cells.

Culture and analysis of minor PBL subpopulations isolated by flow cytometry or immunoadsorption show that a number of other LAK effector cell phenotypes exist (Table 1.3). These variants differ from NK cell-derived LAK cells in their target specificity and generation requirements. Lymphokine-activated killer cells with cytotoxic T cell (CTL) markers ($CD2^{+}3^{+}8^{+}$Leu-$7^{+/-}$Leu-19^{+}) are reported in some studies to be relatively ineffective against freshly isolated tumour cells and the K562 target (38,304,309,310). T cell-derived $CD3^{+}$ LAK cells also require higher concentrations of IL-2 and longer culture times to achieve maximum cytotoxic potential against tumour cell lines. There is convincing evidence to suggest that some $CD3^{+}8^{+}$ LAK cells were

Table 1.3 The surface phenotypes of peripheral blood lymphokine-activated killer (LAK) cells.

Surface phenotype*	LAK activity@
$CD2^{+}3^{-}16^{+}$ Leu-$7^{+/-}$Leu-19^{+}	+++
$CD2^{+}3^{-}16^{-}$Leu-19^{+}	+++
$CD2^{+}3^{+}8^{+}16^{-}$Leu-$7^{+/-}$Leu-19^{+}	++
$CD2^{+}3^{+}4^{-}8^{-}16^{+}$	++

* Presence of surface antigen indicated by +; absence by -; +/- indicates the surface antigen may be either present or absent.

@ Comparative LAK activity against the K562 cell line is graded from + to +++.

originally antigen specific CTL which acquired LAK activity due to further activation by exposure to high concentrations of IL-2 (290,304,311). Indeed, studies of human MLC-derived T cell clones show that alloantigen specific cytotoxicity and LAK activity can coexist in the same T cell (307). Murine experiments support this view and also show that the acquisition of LAK activity by CTL may be reversible (311). Antigen-specific, resting CTL may be capable therefore, of mediating both antigen-specific cytotoxicity and LAK activity after exposure to IL-2 without any further antigen contact (304).

A proportion of T cell-derived LAK cells express the Leu-7 antigen and may therefore originate from $CD3^+Leu\text{-}7^+$ NK cells (284,311). T_γ cells can give rise to LAK cells which bear an identical $CD3^+4^-8^-16^+$ $Leu\text{-}7^-$ surface antigen profile and which are therefore capable of mediating ADCC. Cloned cells of this phenotype express an unusual Ti receptor consisting of one or two γ chains, whereas LAK cells bearing the CD3 and CD4 or CD8 antigens have Ti composed of functional α and β chains (312,313). $Leu\text{-}19^+$ LAK cells which lack both the CD3 and CD16 antigens have not been subjected to further surface marker or genomic analysis to determine their cell lineage (307).

Clonal studies confirm that LAK cells are heterogeneous in terms of their phenotype and cytotoxic activity (292,304,307,308,314). Clones bearing the CD16 antigen generally possessed higher levels of cytotoxicity against tumour cell lines and fresh tumour cells when compared to $CD3^+16^-$ clones. Limiting dilution studies show that approximately 10% of

$CD16^+$ PBL but fewer than 1% of $CD3^+$ precursors gave rise to LAK cells (314). The distribution of the Leu-19 antigen on clones with LAK activity has not been reported, but all such clones, irrespective of phenotype, appear to express the 40 kD CD7 (T40) antigen. Intrathymic and post-thymic T cells and NK cells bear the T40 antigen and the significance of its detection on LAK cells of all types may reflect its expression on most of the known LAK precursor cell types (315). A recently described 120 kD surface epitope designated as LAK-1 has a very similar distribution to CD7. However unlike CD7, LAK-1 appears to participate in the cytolytic process of LAK cells (316).

Precursor cells with a wide variety of phenotypes are capable of giving rise to LAK cells when cultured in the presence of IL-2. The variation in reported phenotypes largely reflects the heterogeneity of LAK precursor cells, but the differing methods used to purify and culture precursor cell populations has also contributed. In the first description of LAK cells, Grimm et al (318) reported that the LAK precursor cells in unsorted PBL were null cells ($CD3^-4^-8^-Leu\text{-}7^-$). However these precursor cells were purified before culture by a series of steps involving antibody and complement-mediated lysis and furthermore, at the time of this study, anti-Leu-11(CD16) antibodies were unavailable. Recent studies have used the more efficient methods of immunoadsorption or flow cytometry to reexamine this issue. There is consensus that the most numerous and efficient LAK precursor cell in the peripheral blood is the $CD16^+$ NK cell (38,60,79,302,309,310,318). The ability to prepare pure samples of

minority subpopulations for LAK cell generation shows that T cells and "null" cells can also act as precursor cells (38,298,309,317,320). It is well documented that $CD8^+$ CTL are LAK precursors, but noncytotoxic $CD8^+$ and $CD4^+$ T cells may also acquire LAK activity in culture with IL-2 (318). Null cells acting as LAK precursors lacked detectable T cell (CD2, CD3), NK cell (CD16, Leu-7) or B cell (CD20) associated antigens (318). The suggestion that even $CD20^+$ B cells develop LAK activity after exposure to IL-2 (317) is supported by another report showing that B lymphoblasts mediate nonspecific cytotoxic activity (319).

The generation of LAK cells from lymphocytes isolated from sites other than the peripheral blood is well documented (14,27,197,317,320). Thymocytes or splenocytes exposed to IL-2 acquire the cytotoxic activity of LAK cells and analysis of the effector cell shows a T cell phenotype (317,320). By contrast, LAK cells derived from human bone marrow lymphocytes are $CD3^-16^+$Leu-19^+ (14). Mucosal lamina propria lymphocytes from the human intestine also contain LAK precursor cells (27,197,321). The phenotypes of LAK precursor and effector cells as determined by antibody and complement-mediated lysis were similar ($CD2^+3^-4^-8^-16^-$ Leu-7^-) and the cells were active against both cell lines and freshly isolated colon carcinoma cells (27,197). More recent studies using panning techniques report that intestinal LAK precursor cells are $CD2^+3^-4^-8^+16^-$Leu-19^+(321). A minority peripheral blood LAK cell population is $CD2^+3^-16^-$ Leu-19^+ and so it is possible that intestinal LAK precursor cells are related to this cell type (309). There is also evidence

from murine tumour models and from clinical studies of malignant melanoma and other solid tumours to show that tumour-infiltrating lymphocyte (TIL) populations contain LAK precursor cells (285,322,323). Lymphocytes infiltrating melanoma tisssue gave rise to LAK cells which expressed a T cell phenotype (285). There is also a suggestion, as yet unconfirmed in human studies, that LAK cells originating from TIL are more potent than their counterparts derived from the peripheral blood (322). It can be concluded therefore, that the LAK cell phenomenon is not restricted to peripheral blood lymphocytes and that LAK cells prepared from extravascular lymphocyte populations may express uncommon or unique phenotypes and cytotoxic properties.

Little is known about the receptors needed to trigger the cytotoxic activity of LAK effector cells. The majority of T cell clones mediating both antigen specific cytotoxicity and nonspecific LAK activity do not utilize their antigen receptor (Ti) or associated CD3 antigen to lyse LAK-susceptible targets (304,307,311,324,325). However, antibodies directed towards the Ti or CD3 antigens significantly inhibit the LAK activity of a few reported T cell clones (293,326-329). In one system, the Ti on $CD3^+$ LAK cells was necessary for recognition of a 120 kD heterodimeric activation antigen on target cells (329). In other studies of $CD3^+$ LAK cell clones however, glycolipid structures on melanoma target cells were bound by receptors unassociated with the Ti (325). Natural killer cell-derived LAK cells appear to possess receptors identical to those of the precursor cell, but in addition, develop novel receptor

populations which are capable of recognizing both NK-sensitive and resistant target cells (304,327). Like NK cell populations, LAK cells derived from heterogeneous precursor populations express a variable, but partially overlapping profile of target receptors (307,327,328). Although some LAK receptors are able to interact with several different targets, there are others which are apparently exclusive for one tumour type (325,327). In the case of T cell clones with dual CTL and LAK activity, the nonspecific adhesion molecule LFA-1 participates in alloreactive cytotoxicity but is not necessary for binding to and lysis of LAK-susceptible targets (308). On the other hand, a recently described LAK cell clone lacking T cell antigens required both surface LFA-1 and LAK-1 antigens for effective cytotoxic activity (316).

Receptor-mediated target recognition by a LAK cell may activate a functionally separate, nonspecific lytic process in the same way as has been demonstrated for virus-specific CTL (330). The nature of the LAK cell cytotoxic apparatus triggered by target cell binding is undefined but it may comprise the same cellular organelles and mediators as utilized by their NK cell and T cell precursors. Stimulation of LAK cells is mediated not only through their target cell receptors but also by cross linking of surface CD2 or CD16 antigens (331). The functional significance of this observation is uncertain, but one possibility is that these structures may function in their own right as both target cell receptors and activation structures.

On a theoretical basis, LAK cells are potentially important

antineoplastic cells, but their presence (as indicated by their characteristic cytotoxic activity) has never been detected in the tumour-infiltrating, mucosal or peripheral blood lymphocyte populations of patients with malignancy (202,27,11). Furthermore, the levels of LAK activity generated in vitro from peripheral blood or intestinal precursor cells are comparable in both normal controls and patients with malignancy (11,27,197,285). This observation suggests that a deficiency of LAK precursor cells does not predispose to the development of neoplasia. Nevertheless, experimental and clinical data dealing with the treatment of advanced malignancy by the adoptive transfer of LAK cells suggest that these cells can be effective against tumours in vivo (111,114,332). It remains to be determined however, whether LAK cells exist spontaneously in vivo in numbers sufficient to influence the initial development or subsequent spread of malignancy.

The LAK cell phenomenon has revived interest in adoptive immunotherapy as a treatment for human cancer. Using leucophoresis and tissue culture methodology, adoptive immunotherapy is being investigated by infusing large numbers of culture-generated autologous LAK cells and IL-2 into patients with disseminated cancer. In both clinical and animal studies, the combination of LAK cells and IL-2 was significantly more effective against established tumours than either therapy administered alone (111,114,332). The human malignancies most susceptible to this protocol were renal cell carcinoma and melanoma. The results of these trials have provoked controversy because the modest rate of complete (8%)

and partial (14%) responses must be balanced against the great expense involved and the serious complication rate. Immunotherapy with LAK cells is an attractive treatment modality with a sound theoretical basis, but many problems have to be addressed before it gains widespread acceptance. Most serious complications are related to the intermittent injection of megaunit doses of IL-2 which can result in acute renal failure and fluid overload with consequent cardiopulmonary failure. Reduction of this adverse effect by continuous infusion of IL-2 has recently been achieved (114). Responses to therapy in many patients are transient, possibly because of the short life span and uncertain tissue distribution of LAK cells after infusion. Although proliferating LAK cells are known to seed to the lungs and liver after infusion in animals, similar data is not available for human studies (333). In the future it is likely that LAK cell immunotherapy will prove most useful in combination with other forms of treatment for malignancies which are resistant to present day conventional therapy.

1.10 Evidence for the participation of natural killer and lymphokine-activated killer cells in nonmalignant human diseases.

Previous descriptions of the antimicrobial and immunoregulatory functions of NK cells would strongly suggest that these cells may play some part in the pathogenesis of diseases where disturbances of immunity are suspected. However, it has proved difficult to convincingly implicate

NK cells in the causation of autoimmune disease (334). Impairment of spontaneous and IFN-inducible NK activity in peripheral blood is a consistent feature of systemic lupus erythematosus (SLE) and similar findings have also been reported in other autoimmune diseases such as inflammatory bowel disease (IBD) and primary biliary cirrhosis (335-337). It has been suggested that an increased frequency of viral infections secondary to reduced NK activity may predispose to the development of SLE and other autoimmune diseases (334). However, apart from this unsubstantiated theory, there is no other plausible role for NK cells in the immunopathogenesis of autoimmune diseases. Indeed the reduction in NK cell function in SLE is generally considered to be secondary to the effects of the disease and its treatment or to recruitment of cells from blood to disease-affected tissue. Even when mononuclear cells have been isolated from diseased tissue (eg intestinal mucosa in inflammatory bowel disease), very little NK activity has been observed (336). Thus, on the basis of the existing evidence, NK cells are unlikely to play an important primary role in autoimmunity.

Antibody-dependent cellular cytotoxicity mediated by lymphocytes may be an important mechanism of organ injury in autoimmune diseases. In studies of autoimmune haemolytic anaemia, K cells mediate ADCC against human erythrocyte targets sensitized with antibodies specific for the blood group antigens (45,338). In autoimmune haemolysis, the density and class of antibody binding to erythrocytes dictates whether their elimination occurs by complement-mediated lysis

and/or clearance by the hepatic and splenic mononuclear phagocytic system. The main mechanism of erythrocyte disposal is probably by opsonization, but ADCC mediated by macrophages and K cells may also be important (45). The human antibody isotypes IgG_1 and IgG_3 are most efficient in mediating ADCC of erythrocytes by both K cells and monocytes. According to recent observations, ADCC may be the main mechanism responsible for clearing sensitized Rh^+ foetal erythrocytes in Rh^- mothers who are given anti-Rh (D) antibody perinatally (45). Moreover, ADCC may contribute to tumour cell eradication in the treatment of malignancy with tumour specific monoclonal antibodies which may not be able to fix human complement (339).

Cells of the NK lineage are also implicated in the spectrum of disorders designated Tγ-lymphoproliferative disease (Tγ-LPD). The hallmark of Tγ-LPD is the finding of excess numbers of large granular lymphocytes (LGL) in the peripheral blood, liver, spleen and bone marrow. It is a rare, indolent lymphoproliferative disorder characterized by hepatosplenomegaly, lymphocytosis, anaemia, neutropaenia and recurrent bacterial infections (340-342). Early attempts to classify the disease using rosetting techniques identified the LGL as T cells bearing Fc receptors for Immunoglobulin G (Tγ cells). More recent analysis of larger series has revealed the heterogeneous nature of this disorder despite the apparent uniformity of LGL (340). Most cases (80-90%) of Tγ-LPD are clonal proliferations of $CD3^+8^+$ T cells which were detected by uniform

rearrangements of the Ti β gene (341). In the remaining cases, non-T NK cells expressing the Leu-7 and CD16 antigens but lacking CD3, are the predominant cell type (342). In a variable proportion of cases, LGL of either T cell or NK cell derivation mediate NK activity and/or ADCC. Evidence from in vitro experiments suggesting that the abnormal LGL can inhibit the formation of haemopoietic colonies provides a plausible explanation for the neutropaenia and anaemia complicating Tγ-LPD (340).

Because the LAK cell was originally a culture-generated phenomenon, there is doubt surrounding its relevance in the normal human or experimental animal. Since LAK cells have no distinctive surface markers, their presence in normal tissue or blood cannot be detected by immunohistochemical methods. Furthermore, under normal conditions, lymphocytes isolated from the peripheral blood, lymphoid organs or mucosal surfaces do not express cytotoxicity characteristic of the LAK cell (11,27). Even after the intermittent infusion of high doses of IL-2, circulating LAK cells were not detected in human subjects, although depletion of LAK precursor cells was reported (112). On the other hand, murine studies have documented the generation of splenic and peritoneal LAK cells during continuous infusion of human recombinant IL-2 in high doses (343).

Although LAK cells cannot be detected under normal physiological conditions, their presence has been demonstrated during immune responses and in disease states characterized by chronic inflammation. During acute infectious mononucleosus, both LAK cells and Epstein Barr virus (EBV)

specific CTL are detectable in the peripheral blood (301). Lymphokine-activated killer cells were reactive against EBV-infected lymphocytes and may thus provide an important accessory function to virus-specific CTL in this and other viral diseases. The potential importance of LAK cells in viral infections is exemplified by a case of fatal infectious mononucleosus where LAK cells could not be generated from the peripheral blood (344). Other pathogens which cause intracellular infections such as the rickettsiae are also susceptible to control by LAK cells (345). During murine intestinal responses to dietary antigens, LAK cells are generated within the intestinal mucosa (346). The presence of potent nonspecific cytotoxic cells in this locality may serve to control the potentially harmful overproliferation of B and T lymphoblasts in the early stages of a specific immune response. Mononuclear cells with spontaneous LAK activity have also been described in Tγ–LPD (342) and after bone marrow transplantation (65). In one reported case of Tγ–LPD, circulating malignant cells with the $CD3^{-}16^{+}$ phenotype mediated LAK activity (342). After bone marrow transplantation, the phenotype of the cells mediating LAK activity was not specified (65).

There is some evidence to suggest that LAK cells can be detected in autoimmune diseases where tissue destruction and chronic inflammation are prominent. Lymphokine-activated killer cells may possess an important primary role in mediating parenchymal cell injury in autoimmune diseases, although it is still uncertain under what conditions

nonmalignant cells become susceptible to LAK activity in vitro (288). Alternatively, LAK cells may arise merely as an epiphenomenon accompanying autoimmune stimulation. In the case of rheumatoid arthritis, $CD2^{+}3^{-}16^{-}$ LAK cells active against K562 cells were found in the synovial fluid from cases of active arthritis, but their cytotoxic activity was not tested against synovial membrane lining cells or fibroblasts (347). Some authors report that freshly isolated lymphocytes and fibroblasts, or endothelial cells in primary culture can be lysed by LAK cells (289-291). Others have found however, that normal peripheral blood lymphocytes are susceptible only after chemical modification of the cell membrane (288). Thus, it is possible that the altered expression of parenchymal cell surface antigens (particularly increased MHC class II antigens) in conditions such as IBD and primary biliary cirrhosis, may predispose to organ damage by LAK cells in vivo (348,349).

From the above data it may be postulated that LAK cells can be generated in the blood or peripheral lymphoid tissues during any immune response which is accompanied by IL-2 secretion. Thus, LAK cells may function as cytotoxic effector or regulatory cells during the amplification stage of any specific systemic or mucosal reaction to microbial or enviromental antigens. Lymphokine-activated killer cells may also arise as a consequence of, and possibly participate in, the abnormal immunological responses which underly autoimmune diseases.

1.11 Cytotoxic cells in the human intestinal mucosa.

The recognition that peripheral blood lymphocytes represent a "transit" population of uncertain relevance to mucosal immunological events stimulated interest in the study of lymphoid cell populations associated with the intestinal mucosa. Because of their accessibility and ease of isolation, lamina propria mononuclear cells (LPMC) have been extensively characterized. Less is known about the functional properties of human intestinal intraepithelial lymphocytes (IEL) as they are difficult to isolate as pure cell populations in sufficient numbers for study. This section reviews the cytotoxic properties of human intestinal lymphocytes and briefly highlights major differences between the cytotoxic systems of the human and rodent intestines. Types of cytotoxicity discussed include NK cell activity, antibody-dependent cellular cytotoxicity (ADCC), lectin-induced cellular cytotoxicity (LICC), LAK cell activity and cytotoxic T cell-mediated lympholysis (CML).

There is general consensus that only very low levels of spontaneous NK activity occur in unfractionated human LPMC populations. When compared with peripheral blood mononuclear cells (PBMC), LPMC have low or absent spontaneous cytotoxicity against the standard NK cell-susceptible K562 target (27,350-354). Because a minority population of cytotoxic cells may be obscured within LPMC populations, techniques designed to specifically isolate NK cells have been applied to the intestinal mucosa. Enrichment of LPMC populations for NK cells by

density gradient centrifugation results in only a small increase in the cytotoxicity of LPMC (27). Amplification of LPMC cytotoxicity by employing very high effector to K562 target ratios in cytotoxicity assays has detected $CD16^+$ $Leu\text{-}7^+$ cytotoxic cells which are probably NK cells (355,356). A recent unconfirmed study using panning techniques to isolate cells from the intestinal mucosa has shown that a minority $CD16^-Leu\text{-}19^+$ LPMC population mediates moderately high levels of spontaneous cytotoxicity against K562 targets (321). The authors propose that these LPMC may be related to infrequent peripheral blood NK cells of similar phenotype (29). In studies of unfractionated LPMC populations, the levels of NK activity were not influenced by the disease process affecting the resected intestine. Hence, LPMC isolated from histologically normal mucosa resected for colorectal carcinoma or nonmalignant conditions and LPMC from actively inflamed mucosae affected by inflammatory bowel disease (IBD) all have comparable NK activity (350,351,353,354,356,357). It was also confirmed by a number of studies that corticosteroid therapy did not alter the levels of intestinal NK activity in IBD (354-356). The results of functional assays for NK cells are confirmed by immunohistochemical surveys of the intestinal mucosa which show very small numbers of cells expressing the Leu-7 or CD16 surface antigens (354,356). The NK activity of LPMC can be significantly increased after treatment with IFN-α or γ, but only by a small increment (27,354,356,357). Although the enzymatic procedure used to isolate LPMC does not inhibit NK activity (27,353,354), prostaglandins liberated during

disaggregation or secreted by intestinal macrophages may be suppressive (358,359). In some authors' hands, incubation of LPMC for 24 h after isolation in the absence of macrophages enhances NK activity (358,359), but others were unable to achieve consistent increases in LPMC NK activity by periods of incubation (27,353) or by the removal of intestinal macrophages (350).

A possible explanation for low intestinal NK activity may be that the standard cell lines used as targets in NK cell assays are not lysed by intestinal NK cells. In view of the proposed antineoplastic and immunoregulatory properties of NK cells, LPMC have been assayed for NK activity against freshly isolated intestinal epithelial and colon carcinoma cells. Single cell suspensions of colonic epithelial cells were reported to be lysed by intestinal lymphoid cells but the uncertain nature of the cytotoxic effector cells and the questionable viability of the target cells flaw these investigations (360,361). A recent study has described the preparation of viable colonic epithelial cell crypts which serve as suitable targets, but as yet, a detailed study of the cytotoxicity of LPMC against these cells has not been reported (362).

The conclusion of a large number of studies is that ADCC against nucleated cell targets is lacking in LPMC populations, irrespective of the underlying disease process (350-352,363-365). Detectable ADCC against nucleated targets was consistently demonstrated in two studies of LPMC, but the levels of cytotoxicity were low (10% specific release or less) (357,366). Significant levels of ADCC are often detected against

antibody-sensitized chicken erythrocytes, but the significance of this observation and the nature of the effector cells is unknown. In PBMC populations, the majority of ADCC is mediated by killer (K) cells which are regarded as a subset of NK cells (50), while Tγ cells also contribute a small proportion of peripheral blood ADCC (16). The minimal ADCC found in the intestine is therefore consistent with the known paucity of NK cells and further indicates that Tγ cells are not plentiful in the intestinal mucosa.

The hallmark of lymphokine-activated killer (LAK) cell activity is nonspecific cytotoxicity directed against cell lines and freshly-isolated tumour cells which are resistant to NK activity (11). Using these criteria, it is evident that LAK cells do not exist spontaneously in the intestinal mucosa in significant numbers (27,197,354). Nevertheless, LAK cells with the capacity to lyse tumour cell lines and colon carcinoma cells can be generated from LPMC by exposure to proliferative doses of IL-2 in vitro (27,197,354,321). These results indicate that precursor cells capable of giving rise to LAK cells are present within LPMC populations. There did not appear to be any gross differences between the frequencies of mucosal LAK precursor cells in colon carcinoma, nonmalignant colonic diseases or IBD (27,197,354).

Lectin-induced cellular cytotoxicity (LICC) is postulated to indicate the presence of primed antigen-specific cytotoxic T lymphocytes (CTL). This type of cytotoxicity is assayed by adding agglutinating doses of specific lectins to potential effector cells and labelled targets in a

short term cytotoxicity assay (367,368). Following the formation of effector and target cell conjugates by nonspecific agglutination, lectin binding to the Ti/CD3 complex activates the CTL and triggers cytotoxicity of targets which are not normally susceptible to lysis. Although NK cells are bound to targets by lectin they do not mediate LICC because they lack Ti/CD3 surface structures (368). CTL may also be stimulated to mediate this nonspecific cytotoxicity by treatment with antibodies directed against CD3 (367,368). Isolated LPMC readily mediate levels of LICC which are usually at least 50% or more of normal PBMC populations (321,350-353,357). As with other forms of cytotoxicity, there was no consistent correlation between the level of LICC and the presence of colon carcinoma or IBD. The ability of LPMC to mediate LICC suggests that primed antigen-specific CTL are present in significant numbers in the intestinal mucosa. Surface marker analysis of LPMC using double labelling studies confirm that from 9 to17% of LPMC express surface antigens associated with CTL (369).

Recent studies of intestinal cytotoxicity have also attempted to identify mucosal CTL on the basis of their antigen specificities. Only one study has so far attempted to generate alloantigen-specific CTL from the lamina propria, but the levels of cytotoxicity achieved were insignificant (370). Convincing evidence indicates that human mucosal CTL specific for intestinal epithelial cell-associated components (ECAC) may participate in the pathogenesis of IBD (371). Preliminary analysis of ECAC reveals four, distinct protein and carbohydrate-containing macromolecules which

are found only on the surface of intestinal epithelial cells. $CD2^+3^+$ LPMC with cytotoxicity directed specifically towards ECAC-labelled targets were detected within inflamed IBD mucosa but not in histologically normal control specimens.

Unlike the diverse mononuclear cell population of the lamina propria, the normal human intestinal epithelium is infiltrated by a uniform population of $CD3^+8^+$ intraepithelial lymphocytes (IEL). Approximately 25% of IEL have cytoplasmic azurophilic granules, but NK cell-associated surface antigens are not expressed on IEL (372,373). Populations of IEL are incapable of NK activity or ADCC, but are able to mediate LICC (351,372,373). It is unknown whether IEL can act as LAK precursor cells, but this possibility is unlikely as exposure to IL-2 did not induce proliferation in cultured IEL (373). The phenotype and cytotoxic properties of IEL suggest therefore that these cells may function as CTL, but as yet, their antigen specificities are completely unknown.

Comparison of human and rodent intestinal mucosal cells reveals striking differences in their cytotoxic properties. Rodent IEL are a complex, heterogeneous population whose origin and function are controversial. Approximately 50% of IEL are thymus-dependent cytotoxic T cells (Thy 1^+ Lyt 1^+ Lyt 2^+) which account for the specific cytotoxicity directed against alloantigens (346,374). A large proportion of the remaining IEL lack Thy 1 and develop independently of the thymus (374). The latter population accounts for most of the granulated IEL but its function is obscure. Cells with NK activity comprise a minority

population within the IEL and are unusual in that they often lack surface antigens found on peripheral blood NK cells (375,376). The levels of NK activity and ADCC mediated by unfractionated rodent IEL populations are comparable to those of splenic or peripheral blood lymphocytes (375-380). Lymphokine-activated killer cells exist in the intestinal epithelium after antigen challenge and specific CTL clones derived from IEL can also be induced to mediate LAK activity by exposure to IL-2 (346,381). Another form of nonspecific spontaneous cytotoxicity mediated by natural cytotoxic (NC) cells is also present in the rodent intestinal epithelium (382). The presence of these cells in lymphoid populations is identified by their capacity to lyse the NK cell-resistant WEHI-164 cell line and their dependency upon interleukin 3 (383). Murine NC cells appear not to be macrophages and they also lack NK or T cell associated membrane antigens. The precise phenotype, distribution and lineage of NC cells is unknown but they are postulated to supplement the cytotoxic functions of NK cells. In a recent study of human NC cells, the evidence suggested that the effector cells may be related to monocytes (384). No studies of human IEL or LPMC have so far tested for the presence of NC cells.

Lymphocytes from the remaining compartments of the rodent intestine have received less attention than the IEL. Peyer's patch lymphocytes represent an interesting population which lacks NK activity but is able to mediate NC (378,382). Lamina propria lymphocytes from the mouse and rat contain a population of functional NK cells which mediate levels of cytotoxicity comparable to IEL (375,379). Like IEL, NK cells from

the lamina propria are unusual in their lack of typical NK cell-associated surface markers (375). Some lamina propria lymphocytes are also CTL, as detected by their ability to specifically recognize and lyse alloantigens on target cells (378).

This section has reviewed the current state of knowledge concerning intestinal cytotoxic lymphocytes. Studies of intestinal cytotoxicity in experimental animals and man reveal an array of different mechanisms active against a diverse range of potential targets. Further investigation is required to confirm the presence of and characterize the infrequent cells mediating spontaneous nonspecific cytotoxicity in the human intestinal lamina propria. In the human, more efficient methods are needed to isolate IEL, so that this population can be made more accessible for further study. The functions of different cytotoxic systems in the normal and diseased intestine are still largely unknown and attempts to implicate aberrant cytotoxicity in the pathogenesis of intestinal disease are in their preliminary stages. In these studies, the important species differences which exist between human and animal intestinal lymphocytes must be considered. Target cells such as intestinal epithelial cells which are more relevant to the study of IBD or colon carcinoma should be used in future investigations. The role of other potentially important cytotoxic cells such as the intestinal macrophage or granulocyte has yet to be examined in any detail. Recently described methods for isolating intestinal macrophages should facilitate these studies (385).

CHAPTER 2.

Intestinal natural killer cells in colon carcinoma and inflammatory bowel disease: an immunohistochemical survey.

I. Introduction

The existence of peripheral blood natural killer cells with antineoplastic (386), antimicrobial (219,220) and immunoregulatory (255,268) properties has prompted searches of mucosa-associated lymphoid tissue for the presence of similar cells (348,350-366,387). Locating intestinal mucosal NK cells is of interest because such cells may protect the normal mucosa from microbial invasion or malignant transformation. Mucosal NK cells may also participate in the disturbed immunoregulation (388) and extensive inflammation and tissue injury (389) found in the intestine during inflammatory bowel disease (IBD). Intestinal lymphocyte populations isolated from cases of colon carcinoma or IBD have uniformly low levels of NK activity against conventional target cell lines (27,350,351). However these results have been questioned because the enzymatic isolation of intestinal lymphocytes may adversely affect their NK activity (358) and may also cause the selective loss of some types of mononuclear cells (390).

The detection of NK cell-associated surface antigens in sections of intestine using immunohistochemistry provides a complementary approach

to functional studies of NK activity in lamina propria mononuclear cells (LPMC) isolated by mucosal disaggregation. The chief attraction of immunohistology is that the number and location of NK cells in both the lamina propria and the epithelial layer of the intestinal mucosa can be determined. Initial investigations of isolated LPMC or mucosal sections demonstrated very few cells expressing the Leu-7 marker which detects approximately 80% of peripheral blood NK activity (28,355,387). Equally low numbers of NK cells have been found in the intestine using antibodies to the Fc receptor for IgG (CD16) which is present on the great majority of NK cells in the peripheral blood (28,354,356,321).

Previous immunohistochemical studies of the intestinal mucosa have been disadvantaged by distortion of tissue morphology attributable to the freezing and fixation methods in use. In a recent study of the human intestine, modified methods of fixing and freezing mucosal sections for immunoperoxidase staining have improved tissue morphology and preserved surface antigens on lymphocytes and macrophages (391). This technique was followed in the present chapter to examine the intestinal mucosa and associated lymphoid tissue for the presence of NK cells using the anti-Leu-7 antibody. The aim of this survey was to investigate possibile abnormalities in the number or distribution of human intestinal NK cells in colon carcinoma and IBD. For this purpose, mucosal specimens from intestines resected for colon carcinoma, IBD or nonmalignant conditions other than IBD were surveyed and compared for the presence and distribution of the Leu-7 cell membrane antigen.

II. Materials and Methods

2.1 Mucosal specimens and patients.

Seven histologically normal specimens of colonic mucosa and one of ileum were obtained at surgery for colon carcinoma (four patients), diverticular disease (two patients) and sigmoid volvulus (one patient). Samples of intestinal mucosa showing active inflammation (seven from the colon and seven from the ileum) were taken from a total of eight patients submitted to surgery for Crohn's disease. Mesenteric lymph nodes from three of these subjects with Crohn's disease were also removed and examined. All the patients with Crohn's disease were receiving corticosteroids (10-40 mg of prednisone daily) at the time of surgery.

2.2 Fixaton and freezing of mucosal specimens.

Within 1 h of resection, specimens of mucosa were cut into strips 1-2mm wide and 5-10mm in length and were fixed for 4 h at 4^{o}C in freshly prepared periodate-lysine-paraformaldehyde fixative (392). The fixative was prepared by adding 2g of paraformaldehyde (dissolved in 25ml of distilled water with 0.05 ml of 10M NaOH heated to 60^{o}C) and 0.214g of sodium-m-periodate to 75ml lysine phosphate buffer. The final solution was then adjusted to pH 7.4 by adding 10M NaOH. Lysine phosphate buffer was prepared by dissolving 18.27g of L-lysine hydrochloride

(Sigma Chemical Co., Mo) in 500 ml of distilled water adjusted to pH 7.4 by the addition of from 150-250 ml of 0.1M Na_2HPO_4 in distilled water. The solution was then increased to a total volume of 1000ml by the addition of 0.1M sodium phospate buffer (prepared by combining four parts of 0.1M Na_2HPO_4 in distilled water and one part of 0.1M NaH_2PO_4 in distilled water). Stock lysine phosphate buffer was stored frozen in 75ml aliquots at -20^{o}C. After fixation the specimens were transferred to a solution of 7% sucrose in phosphate buffer (70g sucrose, 5.68g Na_2HPO_4 and1.56g NaH_2PO_4 in 1000ml distiller water) and were equilibrated with 4 changes of this buffer at 4^{o}C for the next 24 h. The strips of mucosa were then removed from the buffer, blotted to remove excess moisture, and snap frozen at -120^{o}C in the liquified refrigerant Genetron 22^{R} (Monochloro-difluoromethane) (Allied chemicals, Morristown, NJ). The refrigerant gas was liquified by passage through a coil of plastic tubing immersed in liquid nitrogen. The specimens were embedded in O.C.T. compound (Miles Scientific, Naperville, Illinois) and were frozen in stages over dry ice, in liquid nitrogen and then stored at -20^{o}C until sectioned and stained.

2.3 Immunoperoxidase staining.

Immunoperoxidase staining utilized the avidin-biotin-peroxidase (ABC) method (388) with reagents supplied by Vector Laboratories

(Burlingame, California). Tissue sections cut 6-8µm in thickness on a cryostat were placed on ethanol washed glass slides which had been coated in a 0.1% solution of 40,000 MW poly-L-lysine in distilled water (Sigma Chemical Co, Mo) for 10 min and air dried. The sections were air dried briefly on the glass slides before transfer to phosphate buffered saline (PBS).

Staining took place at room temperature in a humidifeid perspex chamber. Sections were initially incubated for 30 min in PBS containing 1.8% normal horse serum and 1% human gamma globulin (Commonwealth Serum Laboratories, Melbourne, Australia) and were then incubated with the primary monoclonal antibody for 2 h. After two 10 min washes in PBS, the endogenous peroxidase activity of granulocytes was neutralized by dehydrating the sections in a brief 60 sec passage through a graded series of solutions of ethanol in water up to 100% ethanol and then treating with 0.1% H_2O_2 in methanol for 10 min. Dehydration was necessary because the presence of water in solution (more than 1%) prevented the inactivation of eosinophil peroxidase activity. Sections were rehydrated, washed and incubated with biotinylated goat anti-mouse antibody (diluted 1 in 200 in PBS with 1.8% normal horse serum) for 1 h and then avidin-biotin-peroxidase complex for 1 h. Specific peroxidase activity was visualized by incubation of the sections in PBS containing 0.5% diaminobenzidine, 10mM imidazole and 0.3% H_2O_2. The sections were washed, lightly counterstained with Mayer's haematoxylin and then mounted under D.P.X. nonaqueous mountant. Control sections were treated

with PBS instead of the primary antibody to detect residual nonspecific peroxidase and nonspecific binding of the biotinylated second antibody or avidin-biotin-peroxidase complex. Photomicrographs were taken with a blue filter to enhance the contrast between the brown peroxidase reaction product and the blue counterstain.

In order to confirm that this method produced positive staining with the Leu-7 monoclonal antibody, normal peripheral blood mononuclear cells (PBMC) were processed in the same way as the tissue sections. Cytospin preparations of PBMC on poly-L-lysine coated slides were fixed by a modified two step procedure at 4^{o}C consisting of incubations in 2% paraformaldehyde with 10mM sodium-m-periodate for 10 min, PBS for 5 min and then lysine phosphate buffer for 10 min. After a 10 min wash in PBS, the PBMC slides were processed in parallel with the tissue sections.

2.4 Monoclonal antibodies.

The anti-Leu-7 monoclonal antibody (Becton-Dickinson, Mountain View, California) was used at the predetermined optimal dilution of 1/20 in PBS with 0.1% human serum albumin (C.S.L., Australia) and 0.1% sodium azide. Optimum detection of $CD4^{+}$ cells was achieved by using a combination of 1/100 OKT4 (Ortho Diagnostic Systems Inc., Raritan, N.J.) and 1/100 anti-Leu-3a (Becton-Dickinson) in the same diluent. Other monclonal antibodies used were 1/20 OKT3, 1/100 OKT8, 1/20 OKT11, anti-Leu-11b in dilutions between 1/10 and 1/1000 and 1/100 25F9 which

was a generous gift from Drs Zwaldo and Sorg of the Universitats Hautklinik, Munster, West Germany (393).

2.5 Quantitation of Leu-7$^+$ cells in the intestinal lamina propria.

Five to six hundred mononuclear cells in inter-crypt areas of the lamina propria were counted under oil immersion. The percentage of peroxidase-positive cells in control sections was subtracted from the value for the anti-Leu-7-treated sections to calculate the actual percentage of Leu-7$^+$ cells.

III. Results

2.1 Normal histology of the ileum and colon.

To assist in interpretation of the mucosal morphology, sections of histologically normal colon and ileum prepared by conventional histological staining methods are shown in Figure 2.1.

2.2 The number and distribution of Leu-7$^+$ cells in the intestinal mucosa.

A mean of 0.5% of LPMC in all fifteen specimens were Leu-7$^+$ (Table 2.1). The positively-staining cells were small to medium-sized

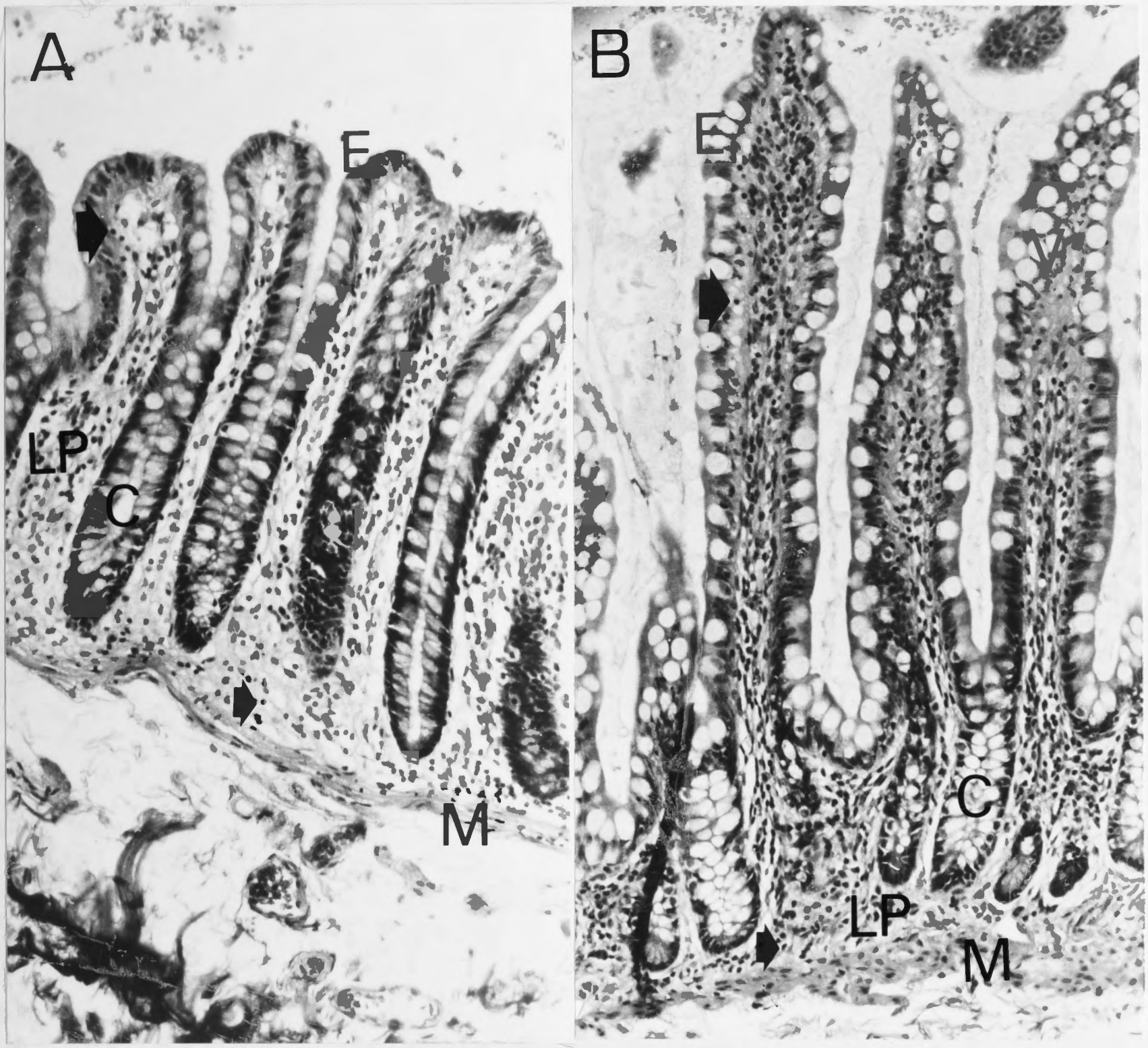

Figure 2.1(A) and (B). Sections of histologically normal colon (A) and ileum (B) from an intestine resected for colon carcinoma. The crypts (C) of the colon and crypts (C) and villi (V) of the ileum are indicated. The lamina propria of the intestine (LP) is bounded on the luminal aspect by the epithelium (E) and on the serosal aspect by the muscularis mucosae (M). Lamina propria (small arrow) and intraepithelial (large arrow) lymphocytes are indicated. (Formalin fixative and paraffin embedding. Haematoxylin and eosin stain, magnification x 175).

Table 2.1 The frequency of Leu-7$^+$ cells in the intestinal lamina propria.

Intestinal disorder*	Frequency of Leu-7$^+$ cells (%)
colon carcinoma (colon)@	0.0%
colon carcinoma (colon)	0.0%
colon carcinoma (ileum)	0.5%
colon carcinoma (colon)	0.7%
diverticular disease (colon)	0.0%
diverticular disease (colon)	1.0%
sigmoid volvulus (colon)	0.6%
Crohn's disease (colon)	0.7%
Crohn's disease (colon)	0.0%
(ileum)	1.5%
Crohn's disease (ileum)	0.0%
Crohn's disease (colon)	0.0%
(ileum)	1.0%
Crohn's disease (colon)	1.0%
(ileum)	0.4%
Crohn's disease (colon)	0.5%
(ileum)	0.2%
Crohn's disease (colon)	0.5%
(ileum)	0.0%
Crohn's disease (colon)	1.0%
(ileum)	1.3%

* All samples of mucosa resected for colon carcinoma or nonmalignant conditions other than Crohn's disease were histologically normal. Mucosal specimens from colon carcinoma were sampled from an area at least 5 cm away from the tumour. All specimens from patients with Crohn's disease showed histological evidence of active inflammation.

@ The source of the mucosa examined is indicated in brackets. In most cases of Crohn's disease both ileal and colonic mucosa from the same resection specimen were examined.

lymphocytes but the density of the membrane staining did not permit identification of cytoplasmic granules. There was no apparent difference between the mean percentages of Leu-7$^+$ LPMC in histologically normal mucosa resected for colon carcinoma (0.3%) or nonmalignant conditions other than IBD (0.5%). Although the specimens affected by Crohn's disease were infiltrated by increased numbers of inflammatory cells, the mean proportion of Leu-7$^+$ cells remained very low (0.6%). The paucity of Leu-7$^+$ cells in representative sections of histologically normal colon and inflamed ileum affected by Crohn's disease is illustrated in Figures 2.2 and 2.3.

Within the lamina propria, Leu-7$^+$ cells were uniformly distributed, with no visible concentrations near the epithelium or the submucosa. None of the intraepithelial lymphocytes in either histologically normal or Crohn's disease mucosa were Leu-7$^+$. Peripheral blood mononuclear cells stained with the same immunoperoxidase method used for intestinal mucosa showed approximately 10% Leu-7$^+$ cells (Fig 2.4). This result agrees with previous phenotypic studies of PBMC and confirms that the method used in this study has adequate sensitivity for the detection of Leu-7$^+$ cells (394).

2.3 The distribution of Leu-7$^+$ cells in mesenteric lymph nodes and mucosal lymphoid tissue.

Mesenteric lymph nodes from three patients with Crohn's disease all

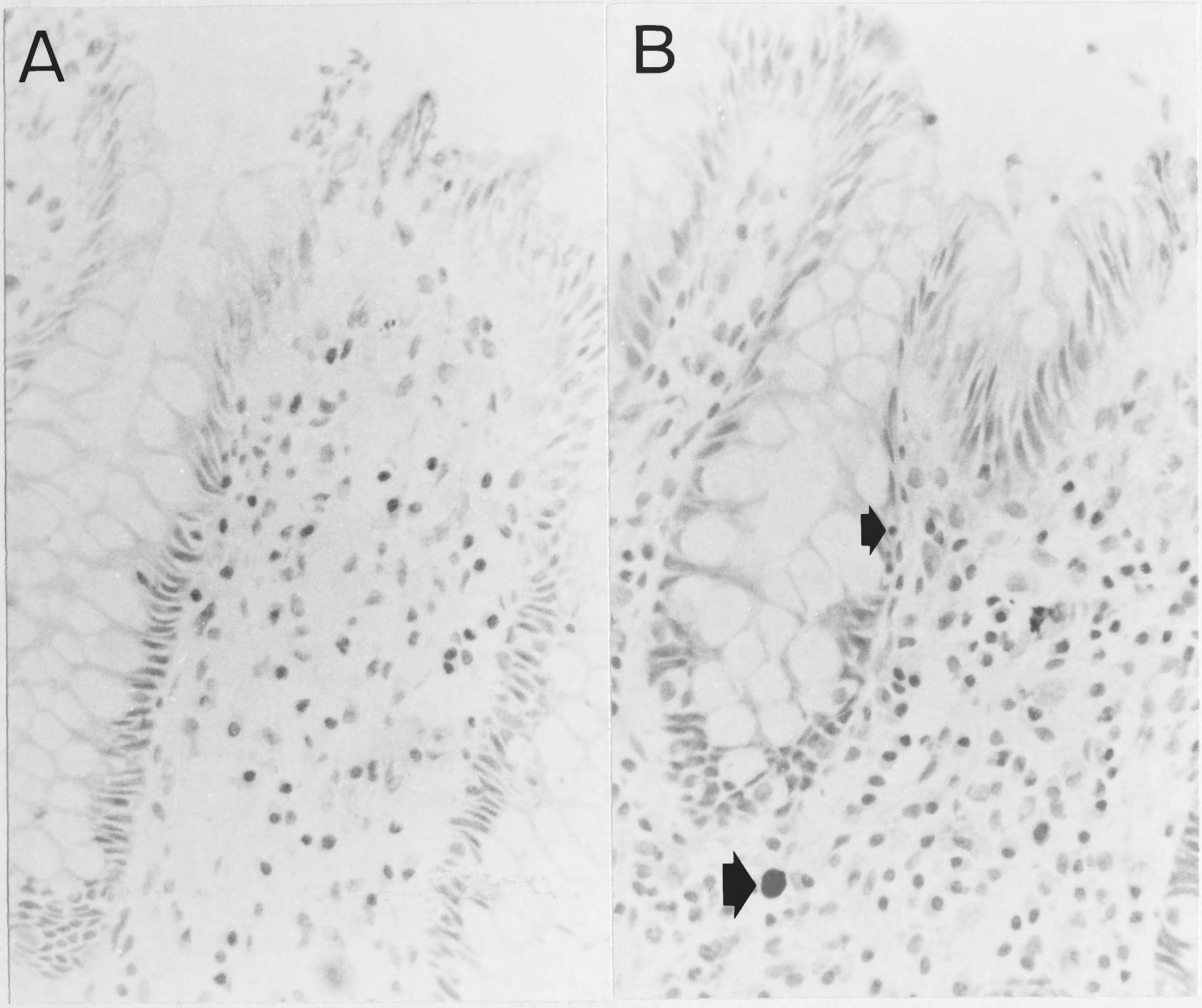

Figure 2.2 Leu-7^{+} cells in the lamina propria of histologically normal colon resected for colon carcinoma. (A) Control section treated with PBS. (B) Anti-Leu-7 treated section. A single positive cell (large arrow) is present in the lamina propria. The intraepithelial lymphocytes (small arrow) are Leu-7^{-}. (Immunoperoxidase stain, magnification x 650).

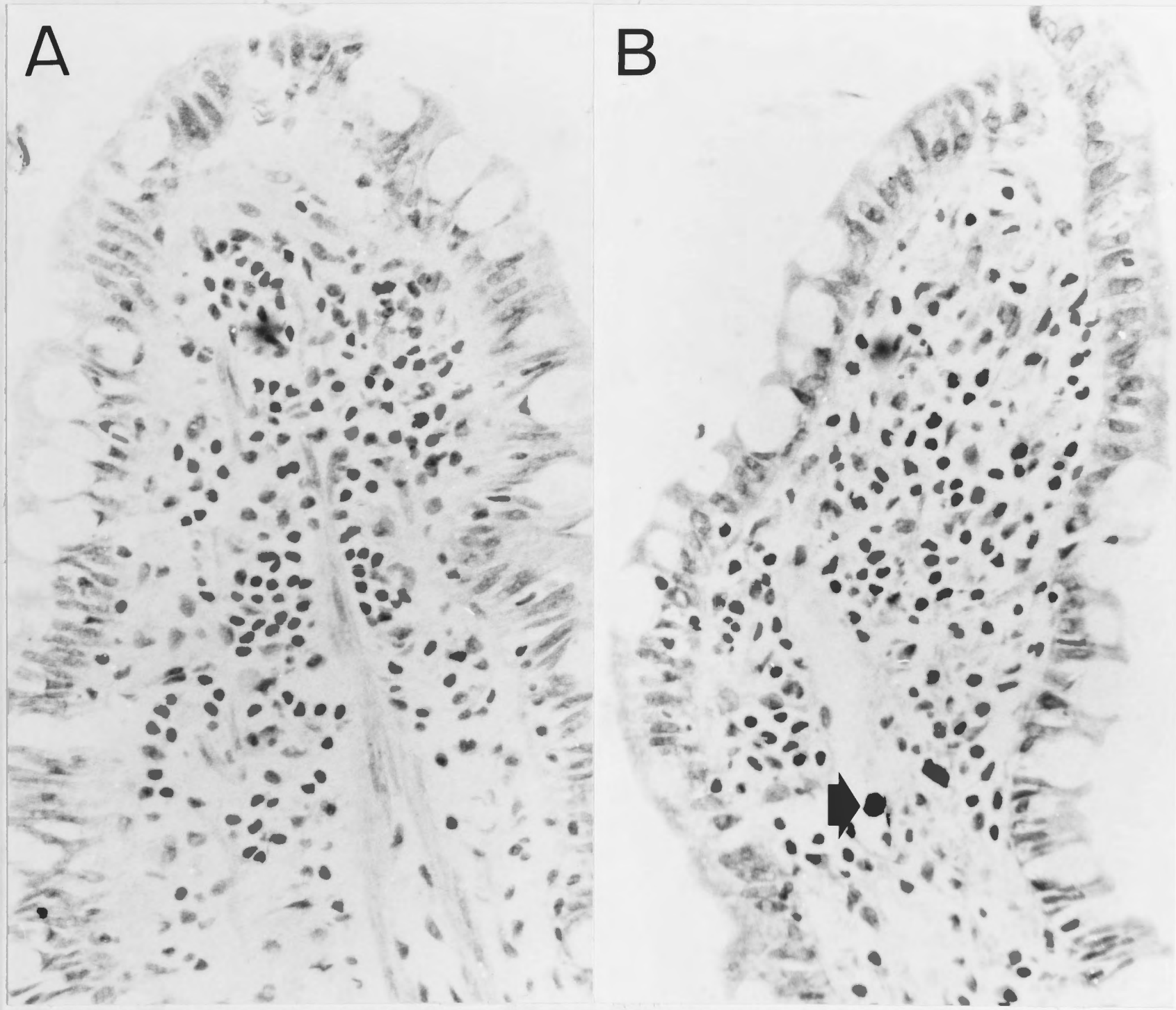

Figure 2.3 Leu-7$^+$ cells in inflamed ileal mucosa resected for Crohn's disease. (A) Control section treated with PBS. (B) Anti-Leu-7 treated section. The mononuclear cell infiltrate in the lamina propria of the villus contains a single Leu-7$^+$ cell (arrow).
(Immunoperoxidase stain, magnification x 650).

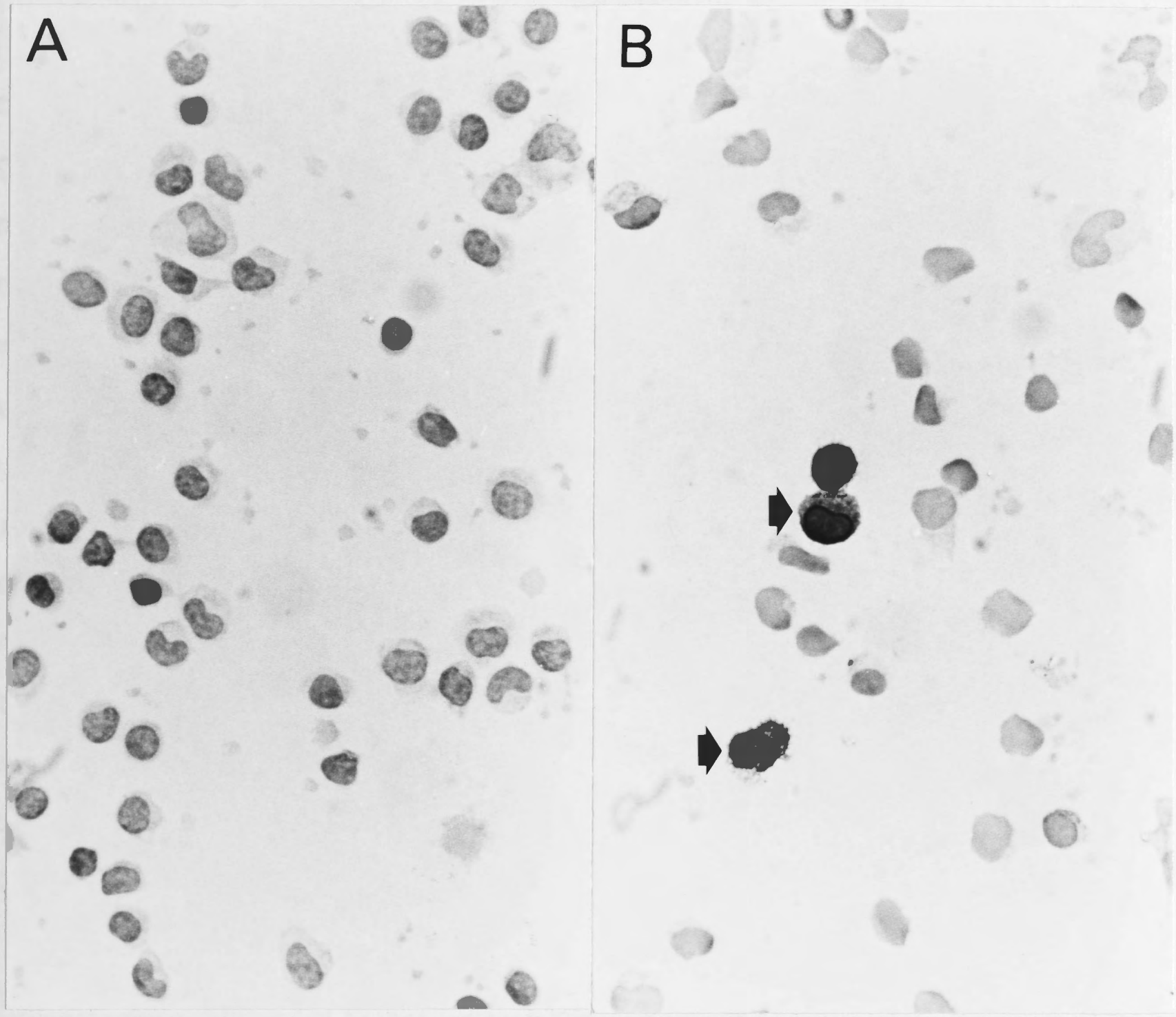

Figure 2.4 Leu-7$^+$ peripheral blood mononuclear cells from a normal donor. (A) Control section treated with PBS. (B) Anti-Leu-7 treated section. The leu-7$^+$ cells (arrows) show the indented nucleus and cytoplasmic granules of large granular lymphocytes. (Immunoperoxidase stain, magnification x 750).

showed a single prominent cluster of Leu-7$^+$ cells within germinal centres (Fig 2.5). These cells had large nuclei and plentiful cytoplasm but intracytoplasmic granules were not visible. A similar staining pattern of germinal centres was obtained using the 25F9 monoclonal antibody which binds to a membrane antigen present on human lung, liver and intestinal macrophages but absent from blood monocytes (391,393) (Fig 2.5). Germinal centre cells positive with 25F9 were approximately the same size as the Leu-7$^+$ cells in the same locality, but the limitations of the staining method precluded further morphological comparison. Outside the germinal centres, small Leu-7$^+$ lymphocytes were scattered sparsely throughout the B and T cell cortical areas and medulla of the lymph nodes. A submucosal lymphoid follicle in the colon and a Peyer's patch in the ileum from two different specimens resected for colon carcinoma also showed clusters of Leu-7$^+$ cells in germinal centres.

Sections of mucosa and mesenteric lymph nodes stained with anti-Leu-11b were uninterpretable due to dense nonspecific staining at dilutions from 1/10 to 1/250 and a lack of specific staining at higher dilutions of the antibody.

2.4 The distribution of T cells and macrophages in the intestinal mucosa and mesenteric lymph nodes.

For the purposes of comparison with the distribution of Leu-7$^+$ cells, the numbers and distribution of T cells and macrophages in the intestine

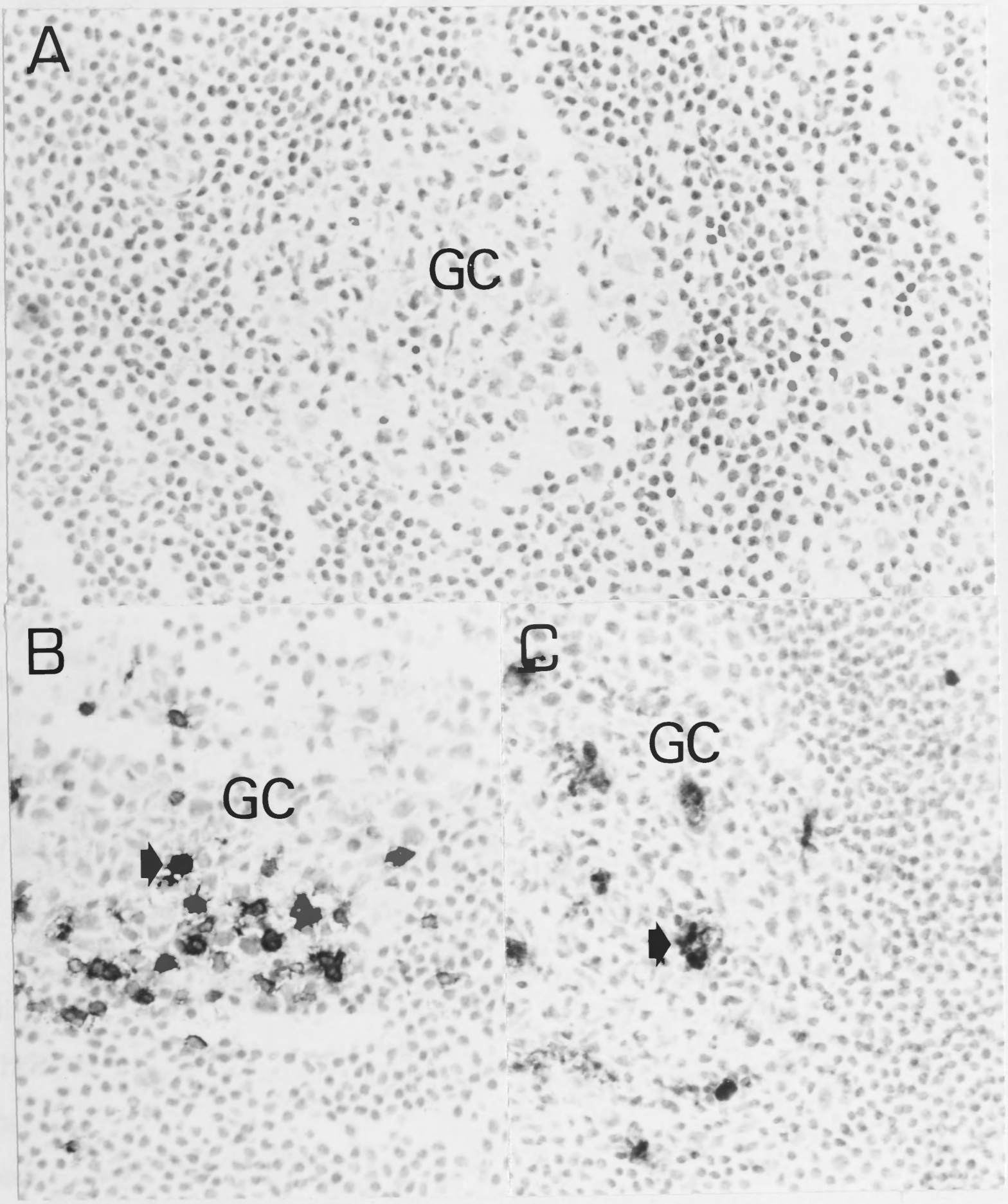

Figure 2.5 Leu-7^{+} and 25F9^{+} cells in the mesenteric lymph node from a patient with Crohn's disease. (A) Control section treated with PBS. (B) Anti-Leu-7 treated section. (C) Anti-25F9 treated section. Staining with either antibody shows positively-staining cells (arrows) clustered within germinal centres (GC).
(Immunoperoxidase stain, magnification x 250).

and mesenteric lymph nodes are shown in representative tissue sections. In agreement with previous studies, $CD4^+$ and $CD8^+$ lymphocytes were located in the lamina propria in a ratio of approximately 2:1 and all intraepithelial lymphocytes were $CD3^+8^+$ (Fig 2.6) (395). Staining the mucosa with the 25F9 antibody identified a population of intestinal macrophages located predominantly in the subepithelial zone of the lamina propria (Fig 2.7). A mesenteric lymph node from a patient with Crohn's disease was stained with the anti-T cell monoclonal antibody OKT3 to delineate the T cell and B cell areas of the node (Fig 2.8).

IV. Discussion

The finding of small numbers of Leu-7^+ cells in LPMC is consistent with all previous studies of the human intestinal mucosa (354,355,387). The Leu-7^+ LPMC population is far outnumbered by intestinal T cells and macrophages and contrasts with the much larger proportion (10-25%) of peripheral blood lymphocytes which bear the Leu-7 surface antigen (394). The Leu-7 marker may not provide a precise reflection of mucosal NK cell numbers as it is absent from a small population of $CD16^+$ peripheral blood NK cells which might also exist in the intestine (28). Moreover, the Leu-7 antigen is also present on subsets of T cells which lack NK activity (396-398). The CD16 antigen is therefore, a more suitable marker for NK cell detection, because it is found only on neutrophils and T_γ cells in addition to NK cells (42,61,119). Technical problems precluded the use of

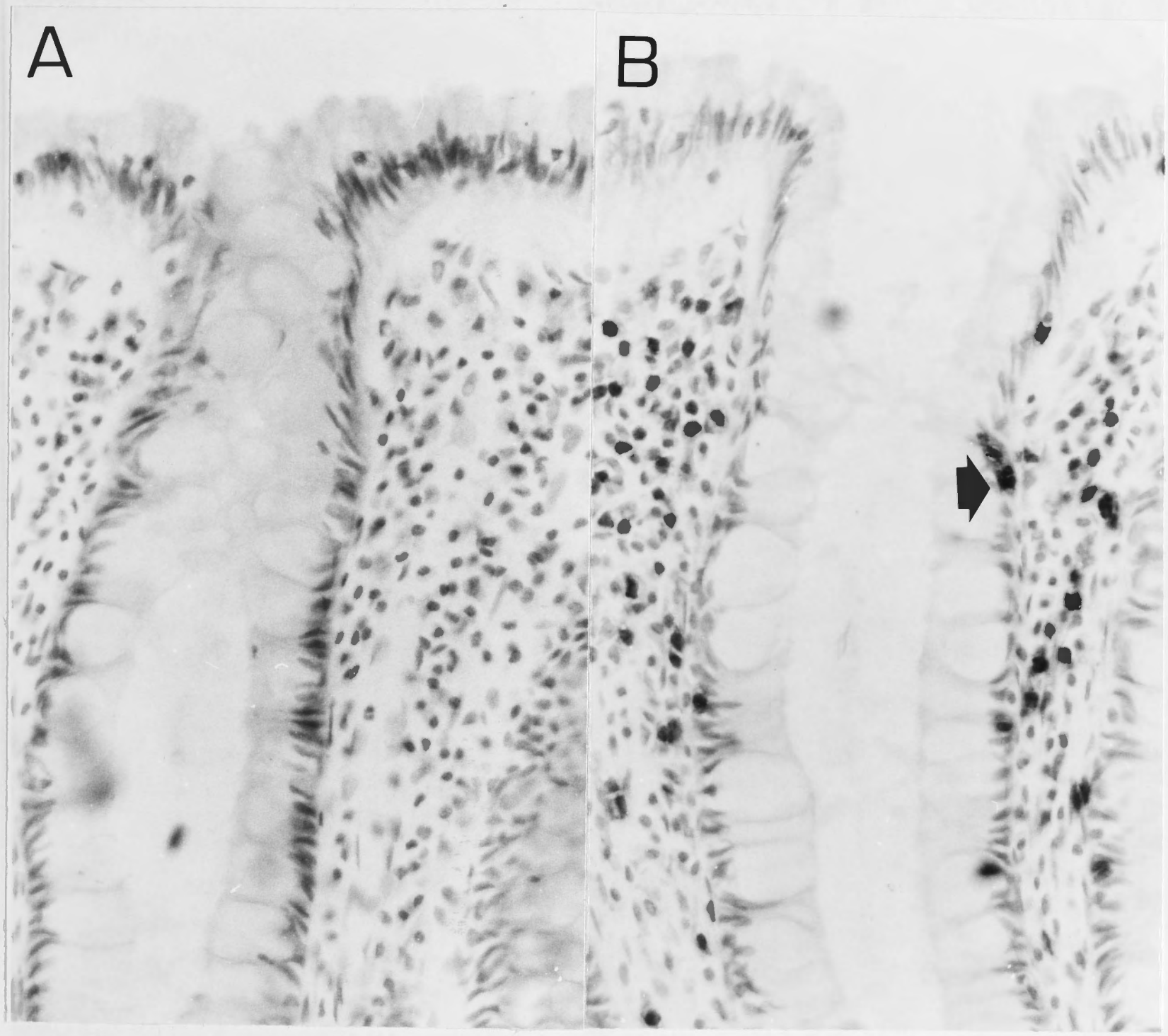

Figure 2.6 T lymphocyte subsets in histologically normal colonic mucosa resected for diverticular disease. (A) Control section treated with PBS. (B) Section treated with OKT3. (see over for figure 2.6, parts C and D).

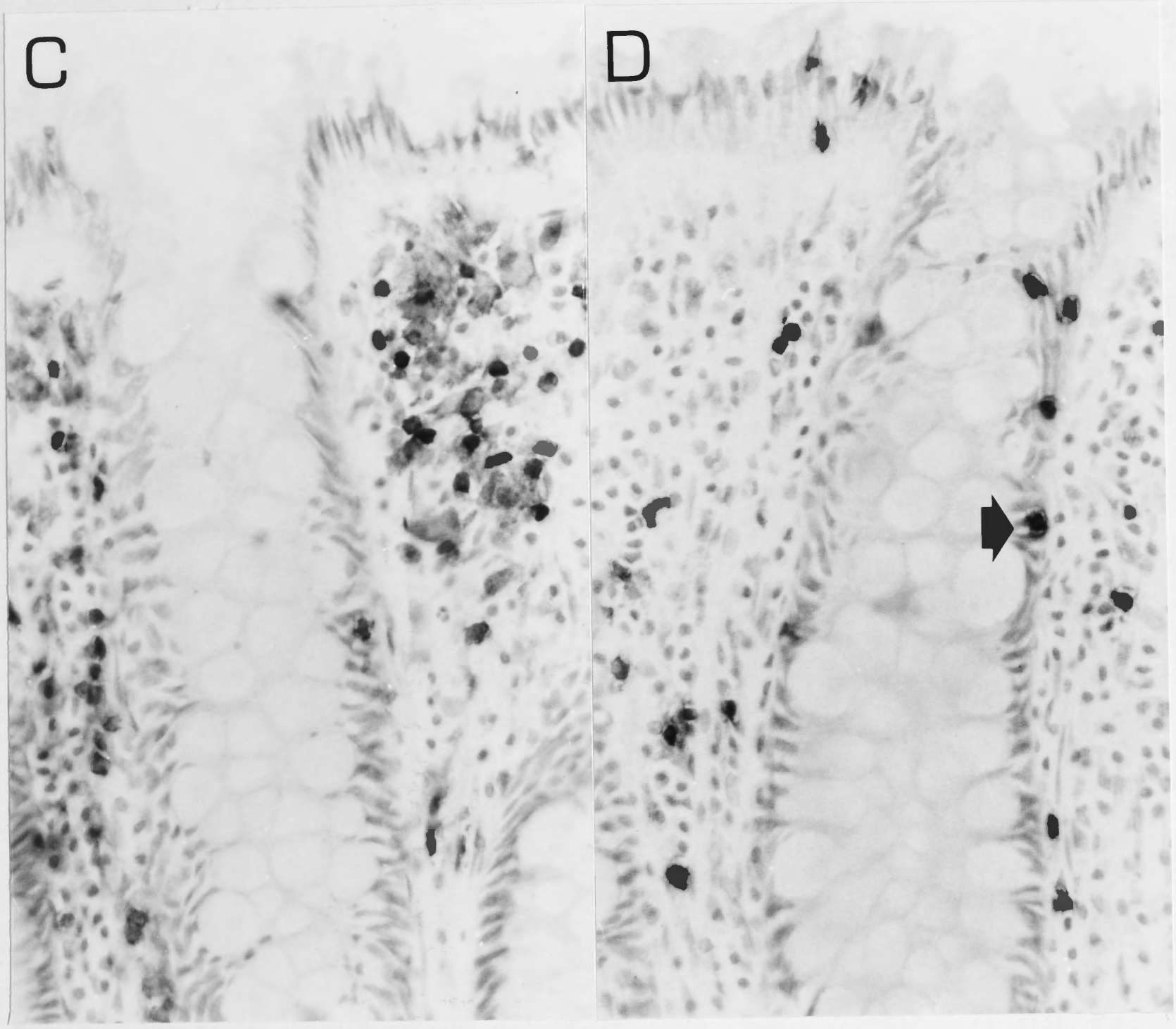

Figure 2.6 (continued). T lymphocyte subsets in histologically normal colonic mucosa resected for diverticular disease. (C) Section treated with OKT4/Leu-3b. (D) Section treated with OKT8. All intraepithelial lymphocytes (arrow) were $CD3^{+}8^{+}$. Lamina propria lymphocytes were mainly $CD3^{+}$ and stained for CD4 or CD8 in a ratio of 2:1. (Immunoperoxidase stain, magnification x 650).

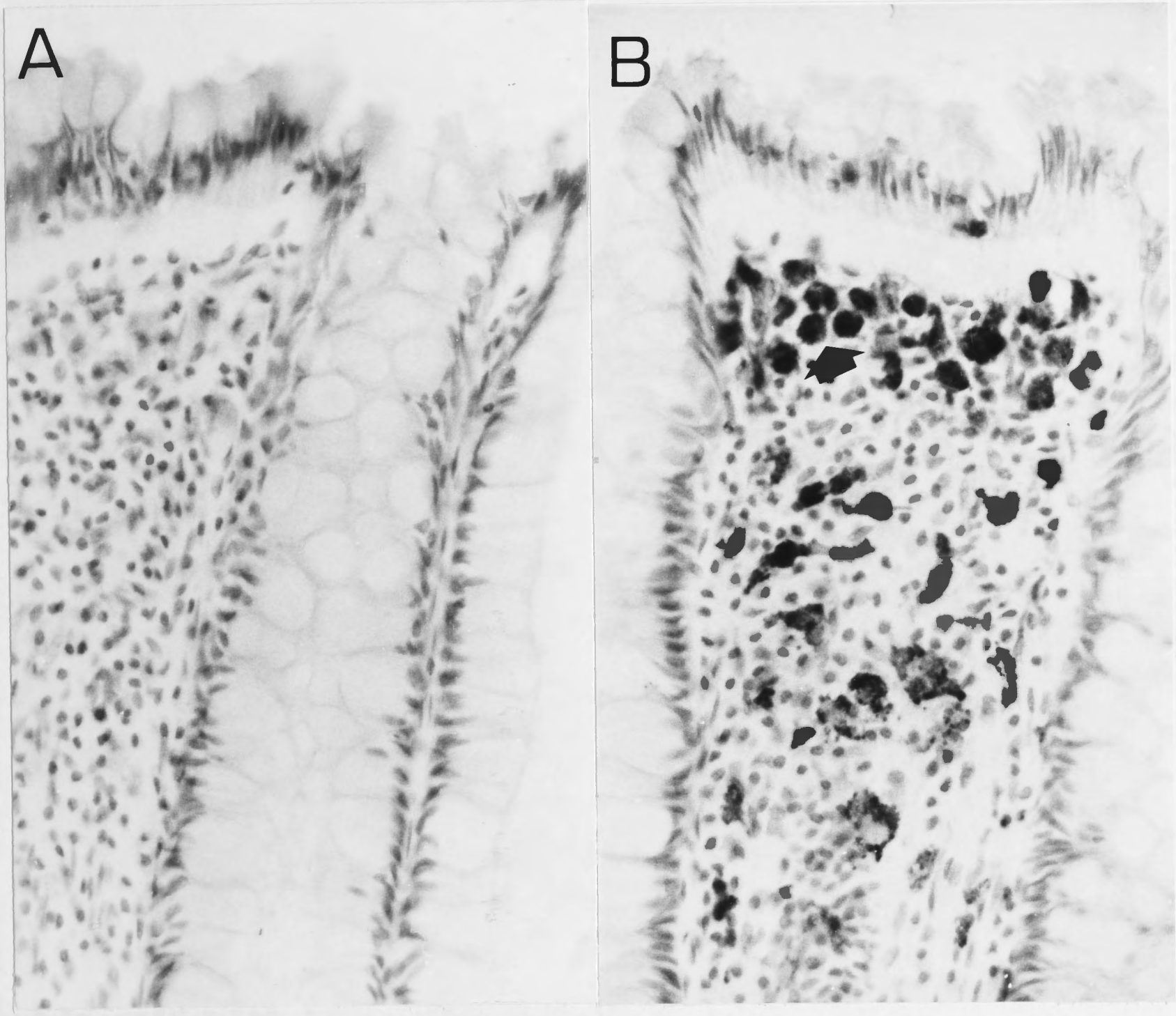

Figure 2.7 Intestinal macrophages in histologically normal intestine resected for colon carcinoma. (A) Control section treated with PBS. (B) 25F9 treated section. Large irregular cells with intracytoplasmic granules are located in the subepithelial zone of the lamina propria (arrow). (Immunoperoxidase stain, magnification x 650).

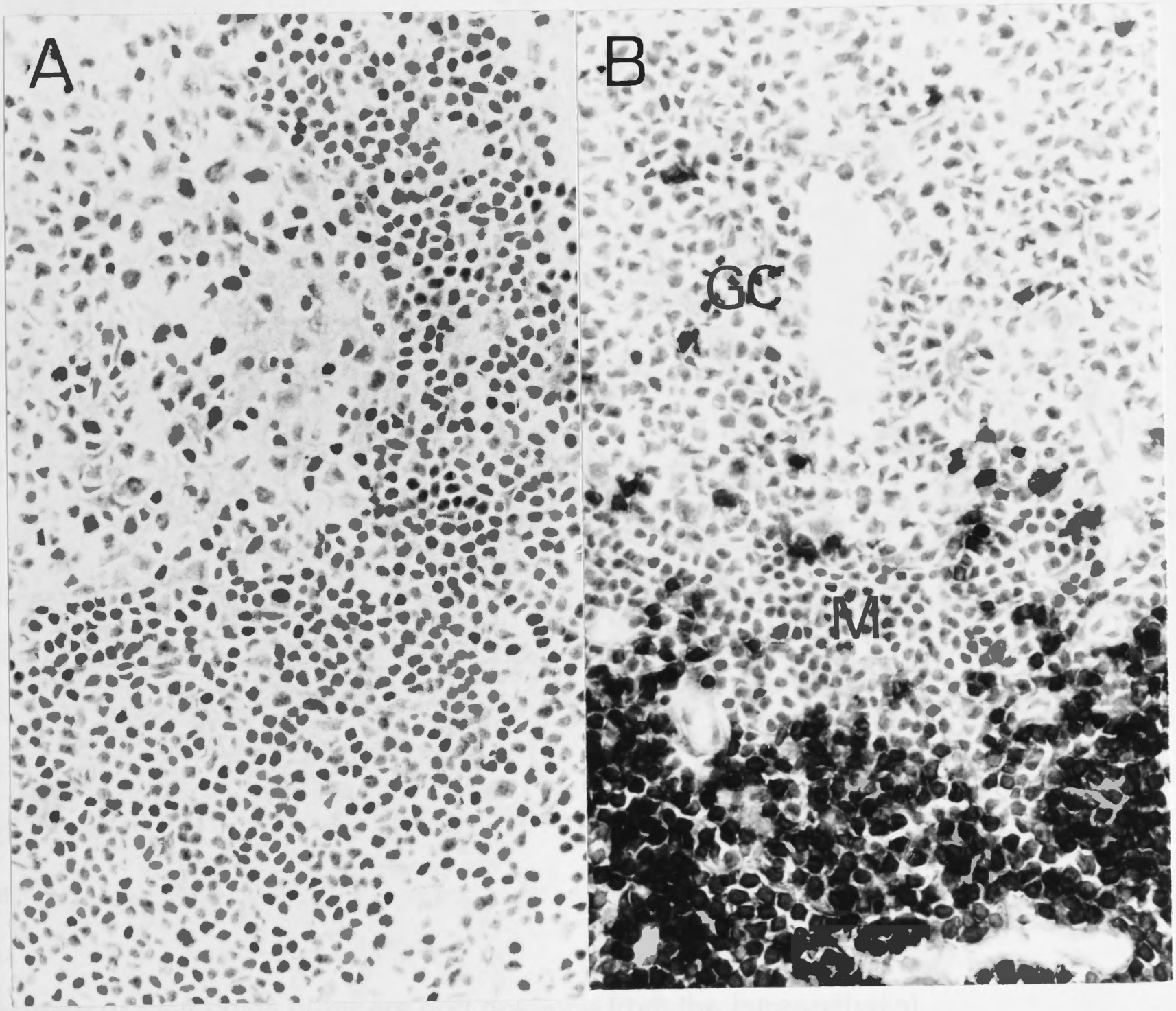

Figure 2.8 The T and B cell areas in the cortex of a mesenteric lymph node from a patient with Crohn's disease. (A) Control section treated with PBS. (B) Section treated with OKT3. The unstained germinal centre (GC) and surrounding mantle (M) of lymphocytes (B cell area) contrast with the surrounding T cells which are $CD3^{+}$.
(Immunoperoxidase stain, magnification x 250).

this marker in the present study, but fluorescent analysis of LPMC suspensions for the CD16 antigen confirm that NK cells bearing this marker are very infrequent (321,356). The paucity of intestinal NK cells demonstrated by immunohistochemistry is thus consistent with low intestinal NK activity which reaches levels comparable to those in the peripheral blood only when tested at very high effector to target ratios (356).

It is difficult to postulate a significant role for NK cells in the normal or diseased intestine using immunohistochemistry because their sparse distribution would make it difficult to detect any subtle change in NK cell number due to disease. A comparison of intestines resected for colon carcinoma or nonmalignant conditions other than IBD showed that there was no discernible alteration in mucosal NK cell numbers in colon carcinoma. These data indicate that there is no gross abnormality of mucosal NK cell numbers associated with established colon carcinoma. Because of their small numbers and absence from the intraepithelial lymphocyte population, the scattered NK cells observed in IBD mucosae are unlikely to be a significant cause of either epithelial cell or connective tissue injury. However, NK cells can exert immunoregulatory effects even when present as a small minority (263,266). Thus it is possible that the immunoregulatory properties of NK cells may be important in certain microenviroments of the normal or inflamed intestinal mucosa despite their small numbers.

The clusters of Leu-7$^+$ cells noted in germinal centers of mesenteric

lymph nodes and mucosal lymphoid follicles have been described previously in the normal human spleen, tonsil and peripheral lymph nodes (277,278,399). Double staining analysis shows that these cells are $CD3^+4^+Leu\text{-}7^+$ granular lymphocytes (278,396). Although rarely found in the normal peripheral blood, these cells are expanded in association with lymphoproliferative disorders such as B cell chronic lymphocytic leukaemia (396-398). Recent studies of cloned $CD4^+Leu\text{-}7^+$ tonsillar cells show that they lack NK activity and do not secrete of IL-2 or B cell growth factor (BCGF) (398). On this basis, germinal centre $CD4^+Leu\text{-}7^+$ lymphocytes differ from $Leu\text{-}7^+$ peripheral blood LGL which modulate B cell activity through the secretion of BCGF (275). Although the function of the $CD4^+Leu\text{-}7^+$ lymphocyte is unknown, their location in germinal centres suggests that they mediate an important immunoregulatory function such as control of isotype switching or isotype-specific T cell help. Both T switch cells and isotype specific T helper cells are $CD4^+$, but their expression of the Leu-7 antigen has not been investigated (400).

The identity of the $25F9^+$ cells in lymph node germinal centres is also uncertain. Without double-labelling studies it cannot be decided whether the Leu-7 and 25F9 markers are carried on the same or different cells. It is likely however, that the $25F9^+$ cells are the prominent 'tingible body' macrophages of the germinal centre (401). The follicular dendritic cell (FDC) also expresses some macrophage-related surface antigens and so is also a candidate for the cell type bearing the 25F9 marker (401).

In conclusion, this study has demonstrated by immunohistochemistry

that cells bearing the NK cell-associated Leu-7 surface antigen comprise a very low proportion of all intestinal mononuclear cells. The possible consequences of these low NK cell numbers with regard to the defence mechanisms of the intestinal mucosa is uncertain. These data cast doubt on the role of NK cell-mediated cytotoxicity in intestinal diseases because of the paucity of identifiable NK cells and also because the number and distribution of Leu-7^+ cells was no different in cases of colon carcinoma, Crohn's disease and other nonmalignant conditions. An unusual Leu-7^+ cell population located in the germinal centres of mucosa-asociated lymphoid tissue may have immunoregulatory functions of relevance to the intestinal mucosa.

CHAPTER 3.

Lymphokine-activated and natural killer cell activity in the human intestinal mucosa.

I. Introduction

Natural killer (NK) and lymphokine-activated killer (LAK) cells may be important components of host defence against neoplasia. Natural killer cells lyse a range of murine and human tumour cell lines in vitro (181,188,189) and selective defects in NK activity in vivo are associated with an increased incidence of spontaneous (188) and experimentally induced tumours in animals (181). Interleukin 2 (IL-2) is essential for both the cytotoxic activity (71) and proliferation (77) of NK cells, whereas interferon (IFN) augments NK activity mainly by increasing the lytic efficiency of functionally immature "pre NK" cells (16).

Lymphokine-activated killer (LAK) cells are a recently described group of cells which also display nonspecific cytotoxicity against tumours, but they can be distinguished from NK cells on the basis of target specificity, kinetics of activation and surface phenotype (11,34,296,300,317,402-404). LAK cells are induced in culture by the proliferation of precursor cells in response to exogenous IL-2 (296) and can also be detected in mitogen or alloantigen-stimulated cultures where IL-2 is plentiful (34,300,403,404).

The functions of NK cells of relevance to the intestinal mucosa are not restricted to nonspecific cytotoxicity as there is considerable evidence to support a regulatory role for NK cells. As well as being IL-2 and IFN-responsive, NK cells release these lymphokines after exposure to viruses, bacteria and mitogens (255,273). Rapidly proliferating lymphoid and haemopoietic cells may be susceptible to control through NK cell-mediated lysis (245,266) and antibody production may also be modulated by NK cells (269). Although peripheral blood LAK cells have been well characterized (11,38,284,309,317) their role in human disease is controversial. The capacity of peripheral blood LAK cells to lyse tumour cell lines and freshly isolated tumour cells suggests an important antineoplastic function (11,403). While the cytotoxicity of peripheral blood NK and LAK cells has been studied in patients with cancer (11,189), the relevance of these activities to local organ involvement by malignancy is uncertain.

Attention has therefore focused on the investigation of local NK activity in intestines resected for colon carcinoma or for nonmalignant disorders (350-353,355,360,361). In all such studies of colon cancer so far reported, spontaneous mucosal NK activity was weak or absent, whereas peripheral blood NK activity in patients with colon carcinoma was readily detectable (353,355). The question now arises therefore, as to whether the LAK cell is the predominant nonspecific cytotoxic cell in the intestinal mucosa.

In the present study, the activities of spontaneous and IFN-inducible

NK cells and of LAK cells were assayed in the lamina propria of histologically normal intestines resected for colon carcinoma or for nonmaligant conditions. In contrast with the low level or absent spontaneous and IFN-inducible NK activity observed, LAK activity against K562 target cells was readily induced by IL-2. These findings indicate that LAK cells may be an important potential source of nonspecific cytotoxicity in the intestinal lamina propria.

II. Materials and Methods

3.1 Patients and specimens.

Sixty-one surgical specimens of intestine obtained from 57 patients were included in this study. Data recorded for patients included: pathological diagnosis, extent of tumour spread according to Duke's classification, histopathological description of intestinal mucosa and relevant drug therapy administered at the time of surgery. There were 40 specimens of colon and five of terminal ileum taken from patients with colorectal carcinoma. Uninvolved mucosa, taken at least five cm away from the carcinoma, was confirmed to be histologically normal in all patients. Fifteen histologically normal specimens of colon and one of ileum were taken from intestine resected for diverticular disease (11 cases), tubulovillous adenoma (one case), sigmoid volvulus (one case), appendiceal abscess (one case), redundant colon (one case) and adhesive

small bowel obstruction (one case).

3.2 Disaggregation of intestinal mucosa.

Full thickness samples of intestine were selected from opened surgical specimens immediately after resection and transported to the laboratory in ice cold RPMI 1640 medium (Gibco, Grand Island Biological Co., N.Y.) supplemented with penicillin (100 IU/ml), streptomycin (100 µg/ml) and neomycin (100 µg/ml). Tissue was then disaggregated as previously described by Golder and Doe (385). Mucosal specimens dissected from muscularis were incubated sequentially in 1mM dithiothreitol (Sigma Chemical Co., St. Louis, MO.) and then 0.75mM EDTA until all epithelial cells were removed. After overnight incubation in medium containing 2U/ml collagenase (CLSPA Worthington, New Jersey, USA); 5U/ml deoxyribonuclease (Calbiochem. Type II, Behring Diagnostics, La Jolla, CA) and 5% human AB serum, lamina propria mononuclear cells (LPMC) were separated from particulate debris by filtration through eight layers of sterile surgical gauze. LPMC were then washed and resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated foetal bovine serum (FBS) (Sterile Systems, Inc.) for density gradient centrifugation.

The average yield of LPMC (mean ± SD) for all specimens was $17.8 \pm 10 \times 10^6$ cells/g mucosa with viability always greater than 95%. Yields from carcinoma and benign conditions were not significantly

different.

3.3 Isolation of peripheral blood mononuclear cells.

Twenty ml of venous blood was obtained by venipuncture from three normal controls and three patients whose colons were later resected for carcinoma. Peripheral blood mononuclear cells (PBMC) were separated by centrifugation over Ficoll-Hypaque gradients (Pharmacia Fine Chemicals, Uppsala, Sweden) and interface cells were harvested, washed twice and resuspended in RPMI 1640 medium with 10% FBS.

3.4 Enrichment of LPMC and PBMC for natural killer cells.

Suspensions of LPMC and PBMC were enriched for NK cells by discontinuous gradient centrifugation using four step gradients of Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) which was a modification of the method of Timonen et al (25). Cell suspensions (75-100 x 10^6 cells in 5 ml medium) were layered over four Percoll fractions consisting of 1 ml of 1.125 g/ml Percoll, 3ml of 1.073 g/ml Percoll, 3ml of 1.063 g/ml and 3 ml of 1.053 g/ml in 15ml plastic centrifuge tubes. Percoll fractions were adjusted to the physiological osmolality of 285 mOsmoles/Kg H_2O.

Gradients were centrifuged at 500g for 30 min at room temperature and then cells were harvested from all interfaces, washed twice and resuspended in medium containing 5% FBS for cytotoxicity testing and

morphological studies.

Measurement of the NK activity of mononuclear cell populations both before and after density gradient centrifugation confirmed that cytotoxicity was significantly enriched for both LPMC and PBMC populations (see results). Maximum NK activity was found in the cell fraction immediately above Percoll density 1.063 g/ml. Therefore all studies described in this section for both LPMC and PBMC utilized cells from this interface.

3.5 Short term culture of LPMC.

Ten LPMC populations (four colon carcinoma, six nonmalignant) were assayed for NK activity both before and after a 24 h period of incubation. LPMC were incubated in 30 ml tissue culture flasks (Lux) at a concentration of 1 x 10^6 cells/ml in 5-10 ml of medium supplemented with 3% AB serum at 37^{o}C in an atmosphere of 5% CO_2 in air. After culture the LPMC were washed and resuspended in medium with 5% FBS for cytotoxicity testing.

3.6 Removal of adherent cells from LPMC.

Seven LPMC specimens resected for carcinoma were assayed for NK activity both before and after removal of plastic-adherent cells. Five ml of the LPMC suspensions (1 x 10^6 cells/ml medium) were incubated in

90mm plastic petri dishes for 60 min at 37^{o}C in an atmosphere of 5% CO_2 in air. Nonadherent cells were removed in four washes with medium and tested for NK activity simultaneously with paired unprocessed LPMC specimens. Nonspecific esterase staining of the nonadherent LPMC showed less than 2% macrophages in all cases.

3.7 Culture of LPMC in MLA144 conditioned medium.

Conditioned medium from the primate lymphoma MLA144 cell line (MLA144 CM) was used as a proven source of IL-2 (408). Filtered MLA144 CM was added at a predetermined optimal dilution to RPMI 1640 and supplemented with 2mM L-glutamine, penicillin (100 IU/ml), streptomycin (100 µg/ml), neomycin (100 µg/ml) and 3% heat-inactivated AB serum and stored at 4^{o}C. LPMC (25 specimens from carcinoma and eight from nonmalignant disorders) were first assayed for NK activity and then incubated at 1 x 10^6 cells/ml MLA144 CM in 30 ml tissue culture flasks (Lux) at 37^{o}C in an atmosphere of 5% CO_2 in air. LPMC from the 1.063 g/ml interface were found to generate maximum cytotoxicity compared with cells from other fractions or unfractionated cells (see results). Thus, the cell populations used to generate LAK cells in this study were from this interface. As a control experiment, nine of these specimens (seven from carcinoma, two nonmalignant) were also cultured under the same conditions in RPMI 1640 medium supplemented only with 3% AB serum and antibiotics. Cytotoxicity against K562 cells was tested at

intervals of up to ten days in culture for all specimens and their paired medium controls. Cytotoxicities recorded as results were the maximum levels of activity assayed during the culture period.

3.8 Treatment of LPMC with anti-Tac antibody.

Four specimens of LPMC from colon carcinoma patients and one sample of PBMC from a normal control were cultured at 1 x 10^6 cells/ml with anti-Tac monoclonal antibody (409) diluted 1/200 with MLA144 CM. Controls included LPMC specimens grown in medium alone or in OKT4 monoclonal antibody diluted 1/200 with MLA144 CM. Cytotoxicity was measured after four days in culture.

3.9 Culture of LPMC in medium supplemented with recombinant IL-2.

Fourteen LPMC specimens (11 from carcinoma and three from nonmalignant disorders) were cultured at 1 x 10^6cells/ml RPMI 1640 medium supplemented with 250 U/ml recombinant human IL-2 (Hoffman-La Roche Inc.,) and 3% AB serum for up to ten days. This concentration of IL-2 was found to be optimal for the generation of LAK activity (see results). Cytotoxicity against K562 cells was then assayed at intervals during culture.

3.10 Culture of intestinal LAK cells in the absence of IL-2.

Eight LPMC populations (five carcinoma, three nonmalignant) cultured in medium containing IL-2 were split at day five into two separate subpopulations. One aliquot of cells remained in the same culture medium with IL-2 whereas the other fraction was washed three times and resuspended at 1 x 10^6 cells/ml in medium supplemented with 3% AB serum but no IL-2. After 24 h, the cultures were terminated and tested for cytotoxicity against K562 cells.

3.11 Incubation of LPMC with interferon.

Triplicate samples of freshly isolated LPMC from six patients (four carcinoma and two nonmalignant disorders) were incubated with 100 U/ml and 250 U/ml of human leucocyte interferon (MRC Research Standard B69/19) in microculture wells (Titertek) at a concentration of 1 x 10^6 cells/ml in 0.1 ml RPMI 1640 and 5% FBS. After incubation for 1 h at 37^{o}C in an atmosphere of 5% CO_2 in air, cytotoxicity was measured leaving IFN in the microculture wells for the duration of the assay.

3.12 Cytotoxicity Assay.

Samples of LPMC and PBMC were tested for NK activity using K562 cells in log phase of growth (407). One million K562 cells were incubated

with 50 µCi sodium ^{51}chromate for 1 h in 0.2 ml RPMI with 5% FBS at 37^{o}C in an atmosphere of 5% CO_2 in air. After four washes, K562 cells were resuspended at 1x10^{5} cells/ml medium supplemented with 5% FBS. Effector cells were assayed in triplicate in 96 well round bottom microculture plates by adding 1x10^{4} target cells to LPMC or PBMC to produce effector to target cell (E:T) ratios ranging from 100 : 1 to 12.5 : 1 in a final volume of 0.2 ml. For spontaneous release controls, aliquots of labelled K562 cells were suspended in medium alone. After 4h incubation, 0.1 ml of supernatant was removed for counting in a gamma counter. Maximum release of ^{51}Cr from K562 cells was calculated by addition in triplicate of 1 x 10^{4} K562 cells in 0.1 ml medium to 0.4 ml 5% Triton X100 (Ajax Chemicals) in 10 ml plastic centrifuge tubes. After 2 h incubation, the detergent solution was centrifuged at 300g for 10 min and 0.25 ml of the supernatant from each tube was removed and placed in 3DT tubes (Disposable Plastics) for counting.

Cytotoxicity was expressed as percent specific Cr^{51} release and was calculated as follows :

$$\% \text{ specific release} = \frac{\text{experimental release - spontaneous release}}{\text{maximum release - spontaneous release}} \times 100$$

The mean spontaneous release expressed as a percentage of maximum release was 12.0% for K562 cells. A specific release value was considered to represent significant cytotoxic activity when it exceeded a cut off level equal to the mean plus three standard deviations of the

spontaneous release controls. This cut off level was 3.0% for K562 cells.

3.13 Phenotyping of intestinal LAK cells using complement-mediated lysis.

Intestinal LAK cells induced by culture in MLA144 CM were phenotyped against K562 target cells by using monoclonal antibody and complement-mediated lysis. Cultures of LAK cells were washed and resuspended in RPMI 1640 with 1% FBS at a concentration of 2.5×10^6 cells/ml. The monoclonal antibodies used were Ortho Pharmaceutical OKT3, OKT4, OKT8, OKT11, OKIa, OKM1 (all diluted 1/50 in medium) and Becton Dickinson Leu-M2 (1/50), Leu-7 (1/50) and Leu-11b (1/20). These dilutions of antibody were shown by fluorescent antibody analysis to produce maximum complement-mediated lysis of the corresponding LPMC subpopulation. The concentrations of Leu-7 and Leu-11b also resulted in maximum reduction of peripheral blood NK activity by antibody and complement-mediated lysis. Ten µl of antibody was added to 2.5×10^4 LPMC in an equal volume of medium in duplicate round bottom microculture wells and stored on ice for 60 min. LPMC were then washed in cold medium, pelleted by centrifugation at 200g for 5 min and after supernatants were removed by needle aspiration the LPMC were resuspended in 20 µl of medium containing a 1/10 dilution of rabbit complement (Cedarlane Laboratories). Following incubation with complement for 60 min at 37^{o}C in an atmosphere of 5% CO_2 in air, a

similar volume of diluted complement was added for a further 30 min. Control cells were treated with medium, antibodies or complement alone. Test and control cells were then washed with medium at room temperature and resuspended in 0.1 ml of RPMI 1640 with 5% FBS. Cytotoxicity of treated and control cells was determined by adding 1×10^4 K562 cells in 0.1 ml to each well.

3.14 Depletion of LAK precursor cells by complement-mediated lysis.

Subpopulations of cells were depleted from four LPMC specimens (all from carcinoma patients) by using complement and each of the antibodies OKT3, OKT4, OKT8, OKT11, OKM1, OKIa, Leu-7 and Leu-11b. The treated LPMC populations were then cultured in MLA144 in order to generate LAK cells. The same procedure outlined above for phenotyping was followed except that 1×10^5 LPMC were treated with 40 µl of monoclonal antibody and 160 µl of diluted complement in duplicate microculture wells. After depletion treatment, LPMC were washed and resuspended at a concentration of 1×10^6 cells/ml in MLA144 CM. Controls included LPMC treated with complement, monoclonal antibody or medium and were cultured in either MLA144 CM or medium alone. Test and control specimens were tested for cytotoxicity against K562 cells after three to eight days in culture.

3.15 Sorting of LPMC by panning.

LPMC from four patients with carcinoma were sorted into $CD2^+$ and $CD3^+$ cells using the OKT11 and OKT3 antibodies respectively. Thirty-five mm x 10mm bacteriological grade plastic Petri dishes (Kayline) were coated with 1 ml of rabbit anti-mouse immunoglobulin (DAKO code 2109) diluted 1/1000 with phosphate buffered saline (PBS) for 40 min at 4^oC. The antibody solution was decanted and the plates were washed four times with PBS, once with 1% FBS in PBS and were stored at 4^oC until used. Samples of LPMC were treated separately with the antibodies OKT3 and OKT11 by incubation of 20×10^6 LPMC in 1 ml of medium containing a 1/50 dilution of either antibody. After 40 min incubation at 4^oC, the treated cells were centrifuged and resuspended in 3.75 ml of Hank's balanced salt solution and 5% FBS. Approximately 4×10^6 cells were poured gently into each of five antibody-coated Petri dishes and were incubated at 4^oC for a total of 70 min with gentle swirling at 40 min to redistribute cells. Nonadherent cells were then harvested from the initial supernatant and in four subsequent gentle washes with PBS and 1% FBS. Adherent cells were removed after a further two washes using a rubber policeman and were resuspended in PBS. Adherent and nonadherent cell populations were washed and resuspended in RPMI 1640 medim for counting. The viabiltiy of both cell populations always exceeded 95%. Cells sorted by panning were cultured in microculture wells for three to eight days at 1×10^6 cells/ml in 0.1 ml MLA144 CM and were then tested

for cytotoxicity against K562 cells.

To detect nonspecific binding of antibody to Fc receptors, LPMC incubated in medium alone were added to antibody coated plates. After incubation and removal of nonadherent cells, direct microscopic examination and counts of adherent cells showed that fewer than 1% of LPMC were binding nonspecifically. Fluorescent analysis of LPMC sorted by panning showed less than 5% contamination with cells of the unwanted phenotype.

3.16 Morphological Studies.

Cell viability was determined by exclusion of 0.125% Trypan Blue. Differential counts were made on methanol-fixed, May Grunwald-Giemsa stained slides prepared in a Shandon cytocentrifuge for 5 min at 500 rpm. The percentage of nonspecific esterase (NSE) positive cells was assessed using α-naphthyl butyrate as substrate as described by Yam et al (405) and Ornstein et al (406).

3.17 Statistical analysis.

Differences between the cytotoxic activities of cell populations were analysed using the Wilcoxon Rank Sum Test. A p value of 0.05 or less was required for significance.

III. Results.

3.1 The NK activity of freshly isolated LPMC and PBMC.

In preliminary experiments, five populations of LPMC and two of PBMC were subjected to discontinuous gradient centrifugation in order to determine which density of mononuclear cells had the maximum NK activity. Analysis of unfractionated cells and the LPMC subpopulations harvested from the Percoll gradient interfaces showed that the cells from the 1.063 g/ml fraction consistently displayed the maximum NK activity (Table 3.1).

The NK activity in this fraction of freshly isolated LPMC was assayed in 34 colon carcinoma specimens and 13 specimens resected for nonmalignant conditions (Fig 3.1). The overall mean activity for all LPMC specimens was 3.4 % at an E:T ratio of 50:1. By contrast, PBMC NK activities from three patients with colon carcinoma and three normal subjects showed a mean activity of 53.7% ($p < 0.01$). No relation was found between LPMC NK activity and the presence or absence of malignancy (mean cytotoxicities were 3.8% and 2.2% respectively) or the extent of carcinoma spread. When the lower limit of significant cytotoxic activity for each assay was set at a value equal to the mean plus three standard deviations of the spontaneous release controls, low level but significant NK cell activity was detectable in 14 specimens (41%) from carcinoma

Table 3.1 The NK and LAK activity of LPMC and PBMC fractions in discontinuous Percoll gradients.

Percoll fraction[a]	NK activity[b]		LAK activity[c]
	LPMC	PBMC	LPMC
Unfractionated	4.3%[d]	40.0%	26.2%
1.053 g/ml	1.9%	43.3%	15.8%
1.063 g/ml	8.2%	66.5%	44.3%
1.073 g/ml	1.9%	24.7%	9.9%
1.125 g/ml	0.0%	15.6%	5.7%

The results shown are representative of five LPMC and two PBMC populations tested.

(a) Cells tested for NK and LAK activity included an unfractionated population before centrifugation and also cells harvested from interfaces above each of the Percoll densities indicated.

(b) LPMC and PBMC from unfractionated and interface populations were tested for spontaneous NK activity immediately after centrifugation.

(c) LPMC from unfractionated and interface populations were cultured in MLA144 CM and then tested for LAK activity.

(d) Percent specific release from K562 cells at an E:T ratio of 50:1.

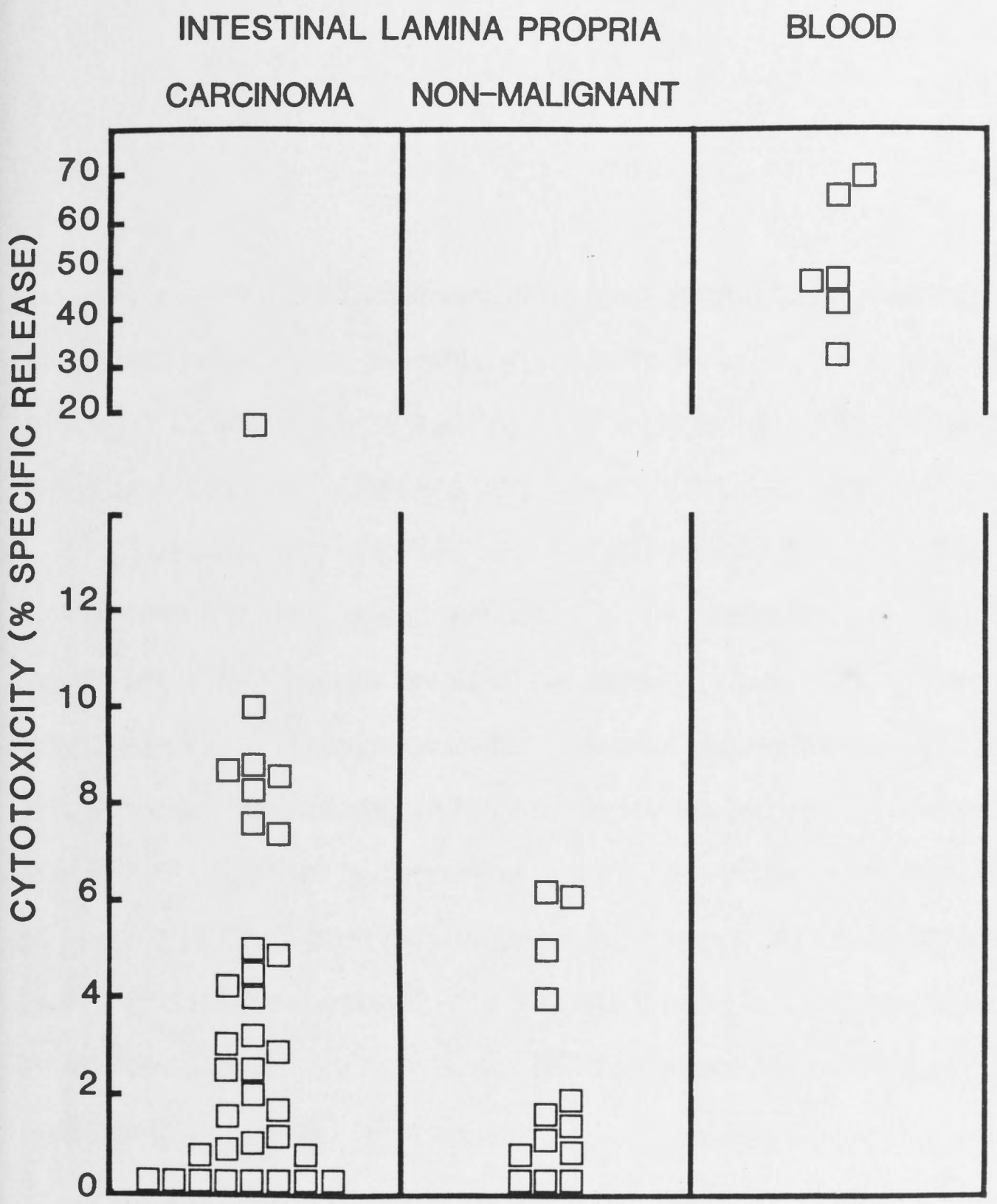

Figure 3.1 Spontaneous NK activity of LPMC from intestines resected for colon carcinoma or nonmalignant conditions. Peripheral blood NK activities in three normal control subjects and three carcinoma patients are included for comparison. The NK activities of the two LPMC subgroups were not significantly different ($p < 0.20$) but the cytotoxicities of both LPMC subgroups were significantly less than that in the peripheral blood ($p < 0.01$). Cytotoxicity is expressed as percent specific release from K562 cells at an E:T ratio of 50:1.

patients and four (30%) from mucosa resected for benign conditions. No significant activity was detected in the remaining 29 specimens. The NK activity of LPMC assayed against K562 cells is therefore absent or detectable at levels which are very low compared to PBMC.

The NK activity of LPMC was not affected by the presence of intestinal macrophages as their depletion by plastic adherence did not significantly alter the low levels of NK activity (Table 3.2). Moreover, incubation of LPMC in medium for 24 h after their isolation did not result in any consistent increase in NK activity for the ten specimens examined (Table 3.3). In order to determine whether the isolation procedure altered NK activity, PBMC from two normal subjects were assayed for cytotoxicity before and after exposure to the treatment used to disaggregate the intestinal mucosa. No substantial difference was found between pre and post treatment PBMC NK activities.

3.2 The effect of IFN on LPMC NK activity.

As the spontaneous NK activity of LPMC was shown to be either low or absent, an attempt was made to augment NK activity using human leucocyte IFN. LPMC from six specimens were tested for NK activity after incubation in either medium alone or in medium containing 100 U/ml of human leucocyte IFN. There was a minor, but significant increase ($p< 0.01$) in mean NK activity from 0.5% to 2.4 % (Table 3.4) , but only one specimen reached significant levels of cytotoxicity. Incubation of two

Table 3.2 The effect of intestinal macrophage depletion on the NK activity of LPMC.

Intestinal disorder	Before depletion[a]	After depletion
colon carcinoma	1.8%[b]	1.2%
colon carcinoma	0.0%	0.0%
colon carcinoma	0.0%	0.0%
colon carcinoma	1.2%	2.0%
colon carcinoma	2.2%	0.9%
colon carcinoma	0.6%	0.0%
colon carcinoma	0.3%	0.0%

(a) Freshly isolated LPMC were tested for spontaneous NK activity before and after depletion of intestinal macrophages by plastic adherence.

(b) Percent specific release from K562 cells at an E:T ratio of 50:1.

Table 3.3 The spontaneous NK activity of LPMC before and after incubation in medium.

Intestinal disorder	0 h incubation[a]	24 h incubation
colon carcinoma	3.2%[b]	1.8%
colon carcinoma	0.0%	0.0%
colon carcinoma	0.4%	0.3%
colon carcinoma	1.2%	4.0%
colon carcinoma	1.0%	0.2%
colon carcinoma	1.0%	2.6%
diverticular disease	0.0%	0.0%
diverticular disease	1.0%	0.4%
diverticular disease	0.4%	3.2%
sigmoid volvulus	0.2%	1.8%

(a) Paired LPMC specimens were tested for spontaneous NK activity immediately upon isolation (0 h) and after incubation in medium for 24 h.

(b) Percent specific release from K562 cells at an E:T ratio of 50:1.

Table 3.4 The effect of IFN on the NK activity of LPMC.

Intestinal disorder	spontaneous NK activity[a]	IFN-augmented NK activity
colon carcinoma	0.2%[b]	2.6%
colon carcinoma	0.2%	3.0%
colon carcinoma	1.0%	2.8%
colon carcinoma	0.8%	1.8%
diverticular disease	0.0%	1.4%
appendiceal abscess	1.0%	2.6%

(a) The NK activity of LPMC was assayed with or without the addition of 100 U/ml IFN.

(b) Percent specific release from K562 cells at an E:T ratio of 50:1.

specimens with concentrations of IFN up to 500 U/ml resulted in no further increase in cytotoxicity. Although the spontaneous NK activity of LPMC was increased by IFN, the levels achieved were still much lower than peripheral blood NK activity ($p< 0.01$).

3.3 The effect of MLA144 CM on LPMC cytotoxicity.

In view of the minimal spontaneous or IFN-inducible lamina propria NK activity, intestinal lymphokine-activated killer (LAK) cell activity was investigated by culturing LPMC in MLA144 CM which is a known source of IL-2 (408). Culture of LPMC from the 1.063 g/ml Percoll fraction produced maximum levels of LAK activity when compared with cells from the other fractions or unfractionated specimens (Table 3.1). After their isolation and assessment for spontaneous NK activity, 1.063 g/ml fraction LPMC from 25 carcinoma specimens and eight nonmalignant specimens were cultured in MLA144 CM for up to ten days and the maximum cytotoxicity against K562 cells was determined. To ensure that increases in cytotoxicity were due to a constituent of the MLA144 CM, nine of the LPMC specimens were also cultured simultaneously in RPMI 1640 medium and 3% AB serum in the absence of MLA144 CM. The cytotoxicity of LPMC cultured in MLA144 CM (mean activity 26.3%) was markedly higher than that of the specimens cultured in medium without MLA144 CM (2.0%) ($p< 0.01$).

As shown in Figure 3.2, the spontaneous NK activity of each LPMC specimen (at time 0) is compared with its maximum LAK activity attained

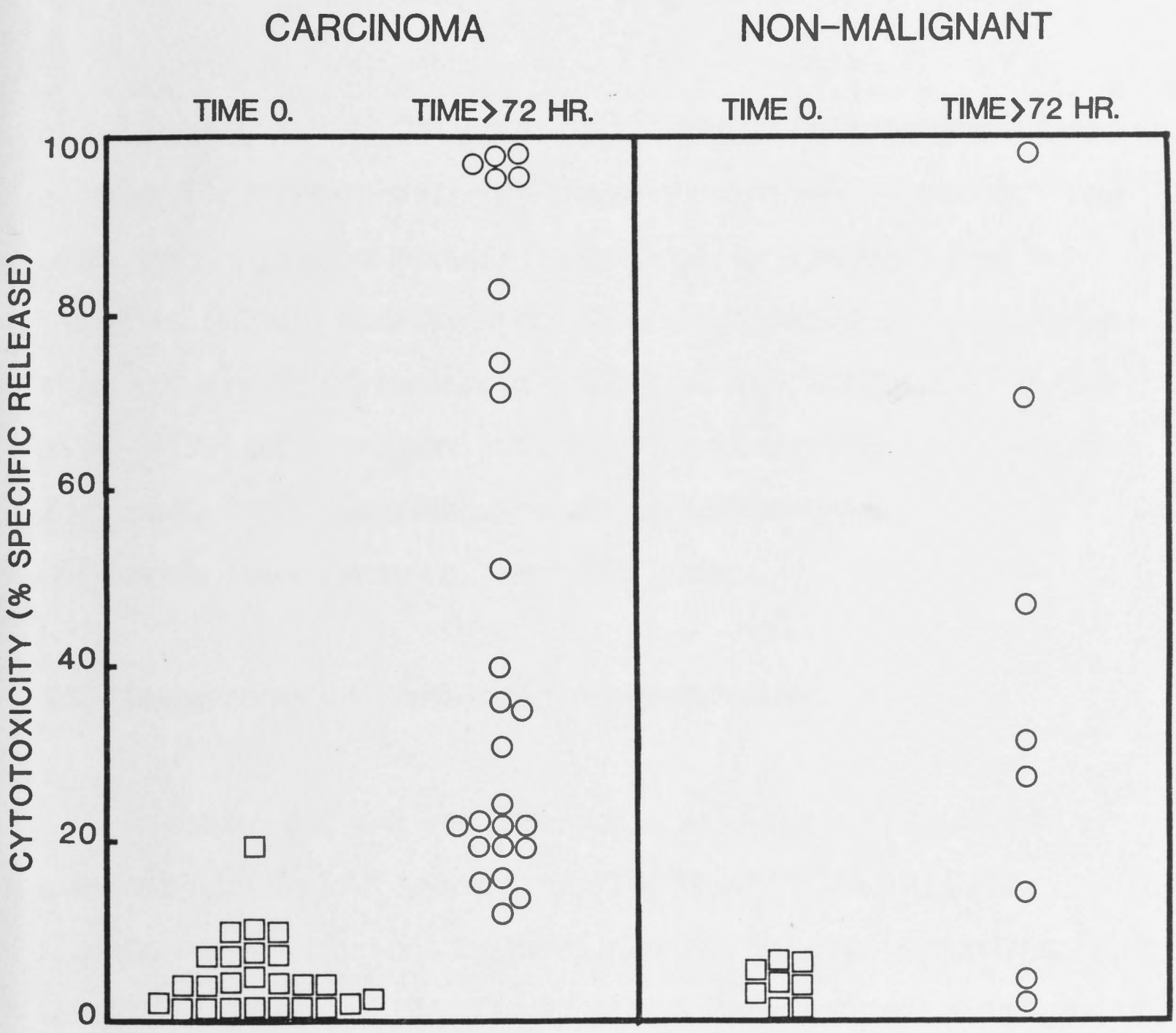

Figure 3.2 The NK and LAK activities of LPMC (25 specimens from carcinoma and eight from nonmalignant disorders) before and after culture in MLA144 CM. The initial spontaneous NK activities (□) at time 0 are compared with the maximum LAK activities (O) attained in culture (> 72 h). The increases in cytotoxicity were significant for both groups ($p < 0.01$ and $p < 0.05$ respectively). Cytotoxicity is expressed as percent specific release from K562 targets at an E:T ratio of 50:1.

after culture in MLA144 CM (time > 72 h). Results from specimens with carcinoma or nonmalignant conditions were considered separately. There was a highly significant increase in cytotoxicity for specimens from both carcinoma (4.3% to 83.6%) and nonmalignant disorders (3.1% to 37.4%) after incubation in IL-2 containing medium ($p < 0.01$ for both groups). The levels of LAK activity induced in malignant and benign specimens were not significantly different and there was also no relation between levels of LAK activity and the extent of carcinoma spread.

3.4 Generation of LAK cells using recombinant human IL-2.

To confirm that IL-2 was the factor, or at least one of the factors, responsible for the LAK activity induced by MLA144 CM, 14 LPMC specimens were cultured in the same manner with human recombinant IL-2 substituted for MLA144 CM. The effect of IL-2 concentration in culture on the development of LAK activity is shown in Figure 3.3. The optimal IL-2 concentration of 250 U/ml was used for the generation of LAK cells. Culture of LPMC in the presence of recombinant IL-2 alone produced high levels of LAK activity (mean activity 91.5% at an E:T ratio of 50:1). LAK activity increased gradually during culture with IL-2, with peak levels attained at six days (Fig 3.4).

The dependence of LAK activity on the presence of IL-2 was studied by withdrawing IL-2 from cultures of LAK cells which had been initiated five days previously. Twenty-four hours after the removal of IL-2, the

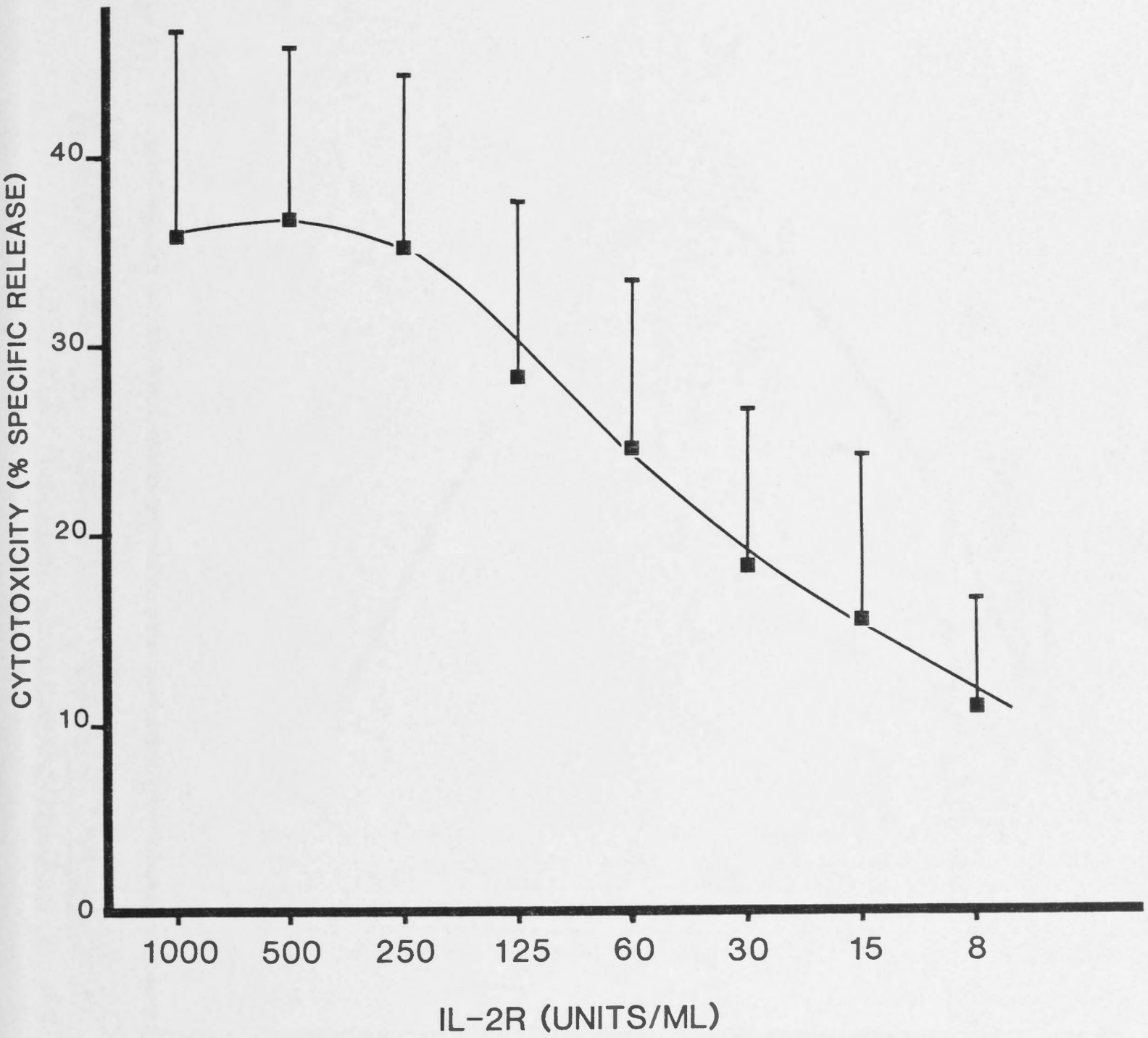

Figure 3.3 The effect of IL-2 concentration on the generation of intestinal LAK activity. LPMC from a specimen resected for carcinoma were cultured with serial dilutions of human recombinant IL-2 in medium for a period of six days and were then assayed for LAK activity against K562 cells at an E:T ratio of 50:1. The vertical bars represent the standard errors of the mean cytotoxicities.

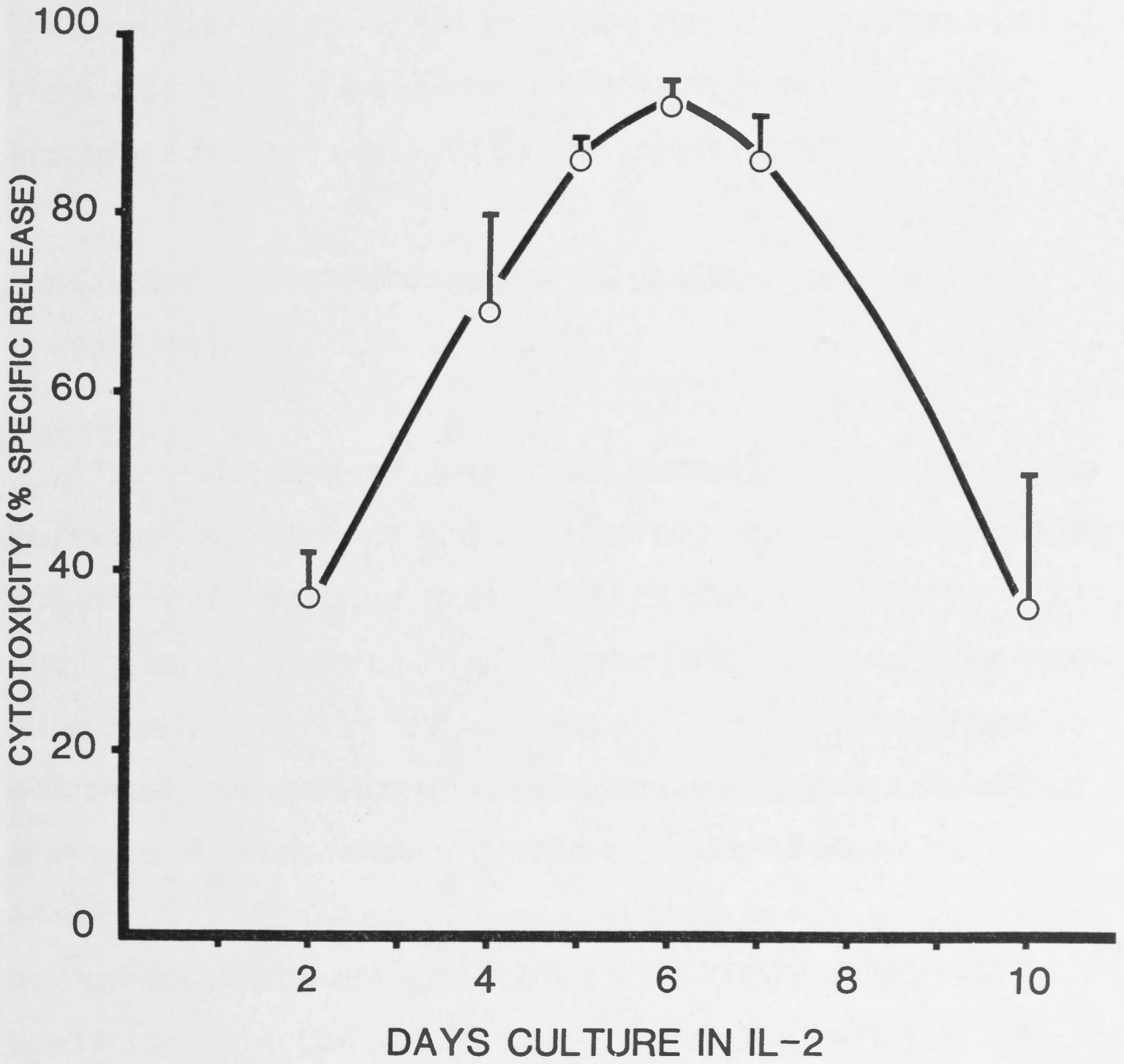

Figure 3.4 The kinetics of intestinal LAK cell activation derived from serial assays of LAK activity during the culture of 14 LPMC populations with human recombinant IL-2. The vertical bars represent the standard errors of the mean cytotoxicities.

cytotoxicity of deprived populations had declined by an average of 40% compared to paired specimens which were maintained in culture with IL-2 (Table 3.5). In four of the seven specimens, the decrease in activity exceeded 30% and the greatest decline noted was 57%.

3.5 Changes in the morphology of LPMC populations after culture in MLA144 CM.

The predominant cell type in freshly isolated LPMC was lymphocytic in appearance, comprising 70% of the total population, while macrophages (identified by NSE staining) represented 17% of the cells. Few lymphoblasts or large granular lymphocytes (LGL) were detected in freshly isolated cells (< 0.5% for both populations). There was no significant difference in the proportion of cell types between specimens resected for carcinoma or for nonmalignant conditions. In order to assess the efficiency of the Percoll gradient system in enriching LGL and depleting macrophages, these cells were counted in the 1.063 g/ml cell fractions used in this study. Compared to unfractionated LPMC cells, macrophages were depleted from 17% to 8%, LGL's were minimally increased from 0.5% to 1% and lymphoblasts were rarely seen (< 0.05%). After LPMC were cultured in MLA144 CM, the proportion of blasts increased markedly to 35%, whereas minimal expansion of the LGL population (2%) was observed (Fig 3.5).

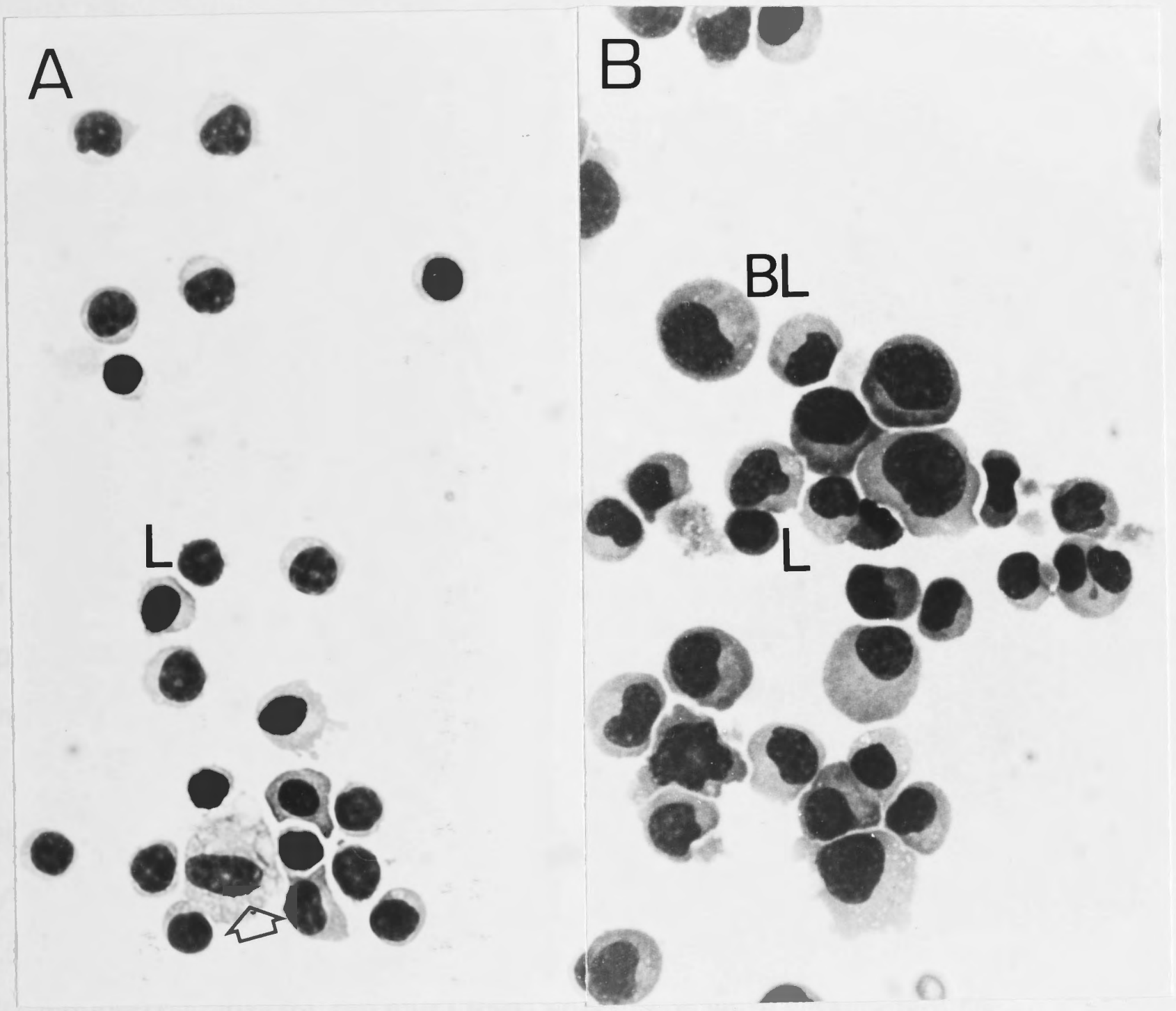

Figure 3.5 (A) LPMC from the 1.063 g/ml Percoll fraction used for assay of NK activity were mainly lymphocytes (L), with a small proportion (8%) of macrophages (arrow), 1% LGL (not easily demonstrable at this magnification), and less than 0.5% blast cells (not shown). (B) Culture of LPMC from this fraction in MLA144 CM generated a heterogenous cell population consisting predominantly of 35% blast cells (BL), lymphocytes (L) and 2% LGL. (May Grunwald-Giemsa stain, magnification x 800 for A and B)

3.6 Effect of the anti-Tac antibody on the generation of intestinal LAK cells.

To test whether the generation of LAK cells was IL-2 dependent, four LPMC specimens from colon carcinoma and PBMC from 1 normal control subject were cultured in MLA144 CM supplemented with a 1/200 dilution of anti-Tac antibody. Anti-Tac is a mouse monoclonal antibody directed specifically against the IL-2 receptor which blocks IL-2 induced proliferation of lymphocytes (409).

Addition of the anti-Tac antibody to cultures of LPMC in MLA144 CM reduced LAK activity against K562 cells in all five cases to levels of cytotoxicity comparable to those of LPMC cultured in medium alone (Table 3.6). To control for a possible nonspecific effect of anti-Tac on MLA144 CM-induced cytotoxicity, the four LPMC specimens were simultaneously cultured with MLA144 CM and an irrelevant antibody of the same isotype (OKT4). The OKT4 antibody is nonmitogenic and defines T helper lymphocytes (410). In three specimens, LAK activity was unaffected, suggesting that anti-Tac specifically blocked the IL-2 receptor. In a further control experiment, the anti-Tac antibody was added to LAK cells immediately before assaying for cytotoxicity. No effect on cytotoxicity was observed, thereby excluding the possibility that cell-bound anti-Tac persisting after washing could inhibit LAK activity.

Table 3.6 The effect of the anti-Tac antibody on MLA144 CM-induced LAK activity.

Intestinal disorder	culture conditions			
	MLA144 CM control	MLA144 CM + anti-Tac	MLA144 CM + OKT4	Medium control
colon carcinoma	19.2%[a]	0.2%	26.5%	0.2%
colon carcinoma	24.4%	0.0%	11.5%	1.0%
colon carcinoma	24.5%	0.2%	29.9%	0.2%
colon carcinoma	41.1%	0.3%	39.5%	3.6%
PBMC (control)	10.6%	0.2%	-[b]	0.6%

(a) Percent specific release from K562 cells at an E:T ratio of 50:1.

(b) Experiment not performed.

3.7 Phenotype of intestinal LAK cells.

Eight LPMC populations (seven carcinoma, one nonmalignant) with MLA144 CM-induced LAK activity against K562 cells were phenotyped by antibody and complement-mediated lysis. During culture in MLA144 CM, multiple assays were performed on individual specimens to ensure that the results were reproducible and representative data from these assays are shown in Table 3.7. In all specimens, treatment with OKT11 and complement reduced LAK activity by at least 30% of control values (complement treatment alone), with average residual cytotoxicity being 30% of control values for all 16 estimations. For the remainder of the monoclonal antibody panel, no consistent reduction in LAK activity was noted. All samples tested were unaffected by the Leu-7 and Leu-11 antibodies which detect 80-85% and 100% of peripheral blood NK activity respectively (28).

Freshly isolated PBMC from two normal controls were enriched for NK cells and were phenotyped in the same way. In contrast to intestinal LAK cells, the NK phenotypes in both cases were $CD2^{+}3^{-}16^{+}Leu\text{-}7^{+}$, as reported by others (28,394,411).

3.8 Intestinal LAK precursor cell phenotype.

The surface phenotype of LPMC required as precursors for LAK cells

Table 3.7 The phenotype of intestinal LAK cells induced by culture in MLA 144 CM.

Intestinal disorder	Monoclonal antibodies[a]								
	C control[b]	OKT3	OKT4	OKT8	OKT11	OKM1	Leu-M2	Leu-7	Leu-11b
carcinoma (1)[c]	26.9[d]	25.0	-[e]	30.2	0.0	-	-	-	22.9
carcinoma (5)	64.4	-	73.4	61.3	33.8	53.5	52.9	72.8	63.9
carcinoma (3)	67.3	58.8	62.8	62.8	46.0	-	67.8	67.9	-
carcinoma (1)	35.7	26.3	31.0	15.7	5.2	27.4	27.1	27.3	-
carcinoma (2)	45.0	36.6	48.7	32.2	14.4	49.8	41.1	46.0	-
carcinoma (2)	17.7	17.7	20.0	22.0	11.3	21.3	24.4	20.0	21.5
carcinoma (1)	12.3	20.8	14.3	13.5	0.0	14.4	15.2	14.8	-
diverticular disease (1)	4.0	8.7	6.4	4.5	2.2	3.9	4.4	4.9	-

(a) Intestinal LAK cells were phenotyped by antibody and complement-mediated lysis using the panel of monoclonal antibodies shown plus complement.

(b) Controls were LPMC treated only with complement before cytotoxicity testing.

(c) The number of experiments performed for each specimen are shown in parentheses. When multiple estimations were done, the single result shown is representative.

(d) Percent specific release from K562 cells.

(e) Experiment not performed.

was determined by complement-mediated lysis and panning methods. Freshly isolated LPMC from three specimens were treated with one of the panel of monoclonal antibodies and complement, and the LAK activity developing in culture with MLA144 CM was then compared with specimens treated with complement alone. For all three specimens, only treatment with OKT11 and complement caused a consistent reduction in LAK activity from a mean control LAK activity of 48.9% to 14.3%. After treatment with any of the other antibodies and complement, no consistent or comparable reductions were noted. When OKT3 was added to LPMC cultured in MLA144 CM or medium alone, a significant increase in cytotoxicity over expected values occurred. This OKT3-induced cytotoxicity may be attributable to the mitogenic properties of this reagent (412) or to the activation of LPMC capable of lectin-induced cellular cytotoxicity (368). The addition of complement alone or any antibody except OKT3 to LPMC before culture in MLA144 CM did not affect the development of LAK activity. To confirm that precursor cells were $CD2^{+}3^{-}$, four LPMC populations sorted into $CD2^{+}$ and $CD3^{+}$ cells were used to generate LAK activity. The mean LAK activity of $CD2^{+}$ cells (74.4%) was markedly higher than that of $CD2^{-}$ cells (46.0%) and the LAK activity of $CD3^{-}$ cells (68.1%) exceeded that of $CD3^{+}$ cells (7.8%) by a factor of nine.

The results from the two previous sections indicate that intestinal LAK cells active against K562 cells are $CD2^{+}3^{-}16^{-}$Leu-7^{-}. Precursor cells required for the generation of LAK activity by IL-2 bear an identical phenotype as established by two different methods.

IV. Discussion.

Previous studies of nonspecific cytotoxic cells in the intestinal mucosa have dealt mainly with NK activity (350-353,355,360,361) which was usually found to be present at low levels or completely absent. In the present study, a novel type of nonspecific cytotoxic cell recently described as the lymphokine-activated killer (LAK) cell has been examined. Intestinal LAK cells with cytotoxicity comparable to, or exceeding peripheral blood NK activity, were produced by exposing LPMC to IL-2 in culture. It was confirmed that IL-2 was responsible for LAK activity by blocking generation of LAK cells with the anti-Tac antibody and also by the induction of LAK activity by using recombinant human IL-2.

The ability of LAK cells to lyse both NK-resistant and sensitive tumour cell lines (403) in addition to freshly isolated tumour cells (11) suggests that they may be important in defence against neoplasia. However the levels of intestinal LAK activity generated by exogenous IL-2 in vitro were comparable in colon carcinoma and nonmalignant disorders. Moreover, no relation was found between LAK activity in vitro and the extent of carcinoma spread. The existence of LAK activity in the intestinal mucosa in vivo may depend upon the availability of IL-2 within the intestinal mucosa. A possible deficit of mucosal LAK (or NK) cells predisposing to malignancy may therefore occur secondarily to defective local endogenous IL-2 production. Studies of IL-2 production by LPMC in vitro report conflicting results, but the bulk of evidence does not indicate

a gross underproduction of mucosal IL-2 in the presence of colon carcinoma (413,414,Chapter 7).

Intestinal LAK cells cytotoxic towards K562 cells were $CD2^{+}3^{-}16^{-}Leu\text{-}7^{-}$ and were generated from lamina propria precursor cells of identical phenotype. This phenotype differs from that reported for peripheral blood LAK cells which are derived from and bear the phenotype of NK or T cells (38,309). Recently a subpopulation of LPMC giving rise to intestinal LAK cells has been phenotyped by Shanahan et al (321) as $CD2^{+}3^{-}8^{+}16^{-}Leu\text{-}19^{+}$. In the present study, the expression of Leu-19 on LAK cell precursors was not examined but they were similar to the cells described by Shanahan et al except for lack of expression of CD8. The phenotypes of intestinal and peripheral blood LAK cells may differ for a number of reasons. First, mononuclear cells from these locations represent different populations as assessed by surface markers (415,416). Unlike PBMC, intestinal lymphoid cells are subjected to sustained antigenic challenge from the luminal surface of the intestinal mucosa. Evidence exists to suggest that this may alter their activation status (385). Comparison is also rendered difficult by the different target cells used as LAK-sensitive targets. Data from the present study are based on the lysis of K562 cells whereas freshly isolated human tumour cells or NK-resistant tumour cell lines were used in the peripheral blood studies (11,300,317). Although intestinal and peripheral blood LAK cell phenotypes differ, their activation kinetics are similar. As was reported for peripheral blood (11), intestinal LAK cell activity was detectable after

two days and increased to a maximum after six days in culture with IL-2. LPMC cultured in IL-2 were a heterogenous population which included a large proportion of lymphoblasts and small numbers of LGL. LAK activity is likely to be mediated by the blast cell population, which is the cell type implicated in peripheral blood LAK activity (300,403). The small numbers of LGL observed, together with the unique phenotype of the intestinal LAK cell, suggest that activation (68) or proliferation (77) of NK cells in IL-2-containing cultures does not account for the cytotoxicity attributed to LAK cells

In contrast to LAK activity, spontaneous NK activity was either present at very much lower levels or totally absent in the intestinal lamina propria. Similar findings using the NK-sensitive K562 cell have been reported previously for LPMC and for lymphocytes infiltrating colon carcinomas (350-353,355). The low or absent NK activity of LPMC populations does not appear to be due to the isolation procedure, as results from the present study and from others (350,353) show that the NK activity of PBMC is unaffected by the enzymatic dissaggregation method. Moreover, incubation of LPMC for 24 h after their isolation did not lead to a consistent improvement in NK activity. It also does not appear that the NK activity of LPMC is suppressed by intestinal macrophages. Although monocytes suppress peripheral blood NK activity (417), it was found in the present study and previously (350) that the presence or absence of intestinal macrophages does not affect the minimal NK activity of LPMC. Indeed, monocytes have been reported to lyse K562 cells (418).

Because NK cells may temporarily adhere to plastic surfaces (21), density gradient centrifugation without prior adherence was used to prepare the LPMC populations examined in this chapter. This procedure resulted in significant depletion of macrophages to 8% of the cell populations used for testing NK activity.

Studies of intestinal NK cells using alternative targets to the K562 cell have also shown reduced or absent NK activity (352,360). In a study using MOLT-4 cells, peripheral blood NK activity far exceeded that of colonic mucosal NK cells. Significant intestinal NK activity against autologous colonic epithelial cells has been reported using a single cell cytotoxicity assay (360) and a microcytotoxicity method (361), but these results are difficult to compare to standard chromium release data.

Results from the present paper and from others (360) indicate only minimal augmentaton of mucosal NK activity by IFN, thus suggesting that "pre-NK" cells are not present in significant numbers in the lamina propria. Morphological analysis of LPMC showed less than 0.5% LGL in freshly isolated suspensions and only 1% LGL in fractions enriched for NK activity. This small proportion of LGL in LPMC is consistent with the low levels of NK activity found. These findings contrast with those using human peripheral blood, where LGL comprise 20% of lymphocytes (419) although only a proportion of these are active NK cells (16). Surveys of human LPMC suspensions (321,355) and mucosal tissue sections (354,387) using the NK-associated markers Leu-7 and CD16 have also confirmed the paucity of these cells in the intestinal mucosa.

In conclusion, the results presented in this chapter indicate that significant LAK activity can be generated in vitro from precursor cells present within the human intestinal mucosa. By virtue of the broad range of susceptible target cells reported for peripheral blood LAK cells and their ready inducibility by IL-2, intestinal LAK cells may have important antineoplastic and immunoregulatory functions in the intestinal mucosa. Further support for the biological role(s) of intestinal LAK cells would be enhanced by experimental evidence for their cytotoxicity against freshly isolated cells from the colonic mucosa. These experiments are described in Chapter 4.

CHAPTER 4.

Intestinal lymphokine-activated killer cells: modulation of their generation and cytotoxic action against colon carcinoma cells.

I. Introduction.

The role of cytotoxic lymphocytes in defence against tumours remains controversial. Both experimental and clinical studies suggest that peripheral blood natural killer (NK) cells may inhibit the development of primary and secondary malignancies (184,192,386). The trend in recent investigations of NK activity in patients with colon carcinoma has been to examine the intestine for evidence of local mucosal NK cell activity (27,354,356). Immunohistochemical surveys of tissue sections and lamina propria mononuclear cell (LPMC) populations isolated from the intestinal mucosa have revealed very low proportions of cells bearing surface markers that are expressed on NK cells (354,356). Furthermore, most studies of isolated LPMC report only minimal levels of NK activity, although this may be in part due to artefacts associated with the isolation procedure (27,354,356,358). The activity of such mucosal cells against freshly isolated tumour cells has not been reported but studies of peripheral blood NK cells have shown fresh tumour cells to be generally resistant to lysis unless the cells are stimulated by interferon or interleukin 2 (IL-2) (11,68,88,303,317). Lymphokine-activated killer

(LAK) cells are a recently described cell population which can be generated from peripheral blood mononuclear cells (PBMC) by stimulation with IL-2 in culture (11,317). Peripheral blood LAK cells exhibit potent non-MHC-restricted cytotoxicity against NK-resistant and susceptible tumour cell lines and freshly isolated tumour cells. Recent studies from our laboratory and from others have demonstrated that LAK cells can be generated from precursor cells contained within LPMC populations isolated from the intestinal mucosa (27,354). As yet, the activity of LAK cells against the most relevant tumour target, the colon cancer cell, has not been examined systematically.

The intestinal LAK cell may require conditions for its generation and activation which are specific to the intestinal mucosa. The critical role of IL-2 in intestinal LAK cell generation has been proven by using the anti-Tac antibody which blocks the IL-2 receptor (27). However the immunomodulating effects of corticosteroids, cyclosporin and interferon on the biology of the intestinal LAK cell has not been reported. Analysis of the effects of these agents is not only of therapeutic interest but may also yield clues to the biological function of LAK cells.

Thus the prime aim of this study was to investigate the possible role of intestinal LAK cells in colonic neoplasia by testing their cytotoxic activity against cells isolated from resected colorectal carcinoma and adenomas. In addition the mechanisms underlying intestinal LAK cell generation and activation were investigated by examining the <u>in vitro</u> effects of mitomycin C, hydrocortisone, cyclosporin and interferon.

II. Materials and Methods.

4.1 Patients and specimens.

Lamina propria mononuclear cells (LPMC) were isolated from 34 samples of histologically normal colonic mucosa obtained from surgical resection specimens. Thirty-three specimens of colon and one of ileum were taken from intestines resected for colonic carcinoma (32 patients); diverticular disease (one patient) and adhesive small bowel obstruction (one patient). In cancer-bearing colons, mucosal specimens were removed from an area at least 5cm distant from the tumour.

4.2 Isolation and primary culture of colon carcinoma and adenoma cells.

Samples of tissue from colon carcinoma or adenoma were washed in phosphate buffered saline to remove bowel contents, trimmed with scissors to remove necrotic tissue, minced finely and then disaggregated by digesting in medium containing 1.2 U/ml Dispase (Boehringer Mannheim, Australia) and 50U/ml collagenase (CLSPA Worthington, USA) and 5% Nu serum (Collaborative Research Inc., Lexington, USA) for 2 hr at 37^{o}C with agitation. The digest was passaged twice through a 20 gauge needle, washed and then dispensed into plastic tissue culture flasks in Dulbecco's Minimal Essential Medium (Flow Laboratories, Virginia, USA) supplemented

with 20% Nu serum; 2% Luria Broth (bactotryptone 10g, yeast extract 5g and NaCl 5g in 1L distilled water adjusted to pH 7.0); 5 ng/ml pentagastrin (I.C.I. Australia); 20mM HEPES buffer; 4mM L-glutamine, penicillin (100 IU/ml) and gentamicin (25 µg/ml). After 1-2 day's incubation at 37°C in an atmosphere of 5% CO_2 in air, viable plastic-adherent tumour cell monolayers were established and could be maintained for up to 30 days using changes of culture medium three times weekly.

When required for use as target cells, adherent tumour cell monolayers were resuspended by exposure for 15 min at 37°C to calcium and magnesium-free Hank's balanced salt solution containing 0.5mM EDTA and 0.2% trypsin. The detached cells were washed twice and resuspended in medium supplemented 5% FBS for labelling with ^{51}Cr. The target cell suspensions contained greater than 80% viable tumour cells (as evidenced by their histological and growth characteristics) with additional small numbers of fibroblasts, lymphocytes and macrophages. If the viability of tumour cells was less than 80%, dead cells were removed before labelling by centrifugation over Ficoll-Hypaque.

The spontaneous release of ^{51}Cr from each target cell expressed as a percentage of maximum release was 31% for colon carcinoma cells, 20% for adenoma cells and 12% for K562 cells. A specific release value was considered to represent significant cytotoxicity when it exceeded a cut off level equal to the mean plus three times the standard deviation of spontaneous releases controls. The cut off levels for significant

cytotoxicity for the target cells were 7.1% (colon carcinoma), 4.6% (adenoma) and 3.0% (K562).

4.3 Phenotypic analysis of intestinal LAK cells.

Three intestinal LAK cell populations from specimens resected for colon carcinoma displaying cytotoxicity towards tumour cells from an adenomatous polyp with carcinoma in situ were phenotyped using antibody and complement-mediated lysis as described previously (27). The monoclonal antibodies used in the dilutions indicated were 1/50 OKT3; 1/50 OKT11 (Ortho Diagnostic Inc. Raritan, NJ) and 1/20 anti-Leu 11b (Becton Dickinson, Mountain View, Ca).

4.4 Cellular proliferation assay.

Six hours before termination of cultures, 1 µCi of tritiated thymidine was added to each microculture well containing LPMC to be tested. LPMC were harvested on Titertek glass fibre filter paper using a Skatron cell harvester (Flow Laboratories, UK). Tritiated thymidine uptake was measured as counts per minute in a Tri Carb 460 liquid scintillation counter (Packard, Illinois, USA).

4.5 Treatment of LPMC with mitomycin C, hydrocortisone and cyclosporin.

Prior to incubation with IL-2, 8 x 10^6 LPMC were treated for 45 min in 2 ml of medium with 50 µg/ml mitomycin C, (Sigma Chemical Co., St. Louis, Mo) and were then washed and resuspended in IL-2-containing medium and cultured in microculture wells. Control cultures of LPMC were not treated with mitomycin C but were otherwise processed in an identical manner. Mitomycin C-treated and control LPMC from triplicate wells were tested daily for cell proliferation and cytotoxicity against K562 targets during the seven days' culture period.

Hydrocortisone (Sigma Chemical Co., St Louis, MO) was dissolved at 2 x 10^{-1}M in absolute ethanol, diluted in medium to a stock solution of 10^{-3}M and titrated in concentrations from 10^{-4} to 10^{-10}M into triplicate microculture wells containing LPMC at the commencement of culture with IL-2. Addition of equivalent concentrations of ethanol to cultures of LPMC with IL-2 had no effect on the generation of LAK activity (data not shown).

Cyclosporin was a gift from Sandoz Ltd (Basel, Switzerland) and was prepared as a stock solution of 50 µg/ml in medium as described by Warren et al (428). Cyclosporin in concentrations of 0.1 µg/ml and 0.3 µg/ml was added to LPMC at the beginning of cultures with IL-2. Both hydrocortisone and cyclosporin remained in the cultures until they were terminated for testing. Untreated LPMC in culture with IL-2 were included as controls for each agent tested. After six days' culture, treated and

control LPMC were washed twice in the microculture wells and then tested for cytotoxicity against K562 cells.

The effects of hydrocortisone and cyclosporin were also tested on two freshly isolated and unfractionated LPMC populations cultured either with a mitogenic dose of phytohaemagglutinin (PHA) (Commonwealth Serum Laboratories, Melbourne, Australia) or in mixed lymphocyte culture (MLC). For mitogen stimulation, LPMC were cultured for four days at a concentration of 1×10^6 /ml in microculture wells in 0.1 ml of medium supplemented with 3% AB serum and 20 µg/ml PHA. Mixed lymphocyte cultures of ten days' duration were performed in microculture wells by mixing 5×10^4 LPMC with an equal number of mitomycin C-treated PBMC from a combined pool of five normal donors in 0.2 ml of medium supplemented with 3% AB serum. Hydrocortisone and cyclosporin in the concentrations outlined above were present at commencement and throughout the period of culture. Upon completion, the proliferative activity of treated cultures was compared with untreated controls.

4.6 Treatment of intestinal LAK cells with interferon.

Intestinal LAK cells generated after five to eight days' culture of LPMC with IL-2 were tested for cytotoxic activity against K562 cells both before and after treatment with human leucocyte interferon (IFN) (Medical Research Council, UK Reference Standard B69/19). Triplicate samples of LAK cells were incubated with 125 U/ml and 250U/ml of IFN for 1 h prior

to and during the cytotoxicity assay against K562 cells at an E:T ratio of 50:1.

Details of LPMC and PBMC isolation, NK cell enrichment, the cytotoxicity assay and statistical analysis are given in Chapter 3. Lymphokine-activated killer cells were generated using 250 U/ml human recombinant IL-2 as described in Chapter 3.

III. Results

4.1 Intestinal LAK cell activity against colon carcinoma cells.

As previously shown (27), LAK cells generated by culture of LPMC with 250U/ml recombinant human IL-2 develop maximum cytotoxicity against K562 cells after six days. In the present study, intestinal LAK cells induced under identical conditions were tested for their ability to lyse freshly isolated colon carcinoma cells. Significant levels of LAK cell activity against colon carcinoma cells were demonstrated in seven of eight specimens tested (Table 4.1). In the single LAK cell population lacking significant activity, lysis of K562 cells was also low. Lysis was not restricted to autologous combinations of effector and target cells, as the colon carcinoma cells were lysed by LAK cells prepared from either the same or different specimens. Parallel cultures of LPMC in the absence of IL-2 failed to develop cytotoxicity against colon carcinoma or K562 targets (data not shown).

Table 4.1 Cytotoxicity of intestinal LAK cells against colon carcinoma cells

Indication for intestinal resection	Target cells	
	colon carcinoma	K562
carcinoma	11.9%(a)	42.4%
carcinoma	19.7%	41.7%
carcinoma	48.4%	76.4%
carcinoma (autologous)(b)	0.2%	18.6%
carcinoma (autologous)	35.7%	52.3%
carcinoma (autologous)	12.4%	55.5%
carcinoma (autologous)	27.3%	66.6%
carcinoma (c)	14.4%	82.0%
carcinoma (c)(autologous)	25.4%	82.0%

(a)percent specific release from target cells at an E:T ratio of 50:1.

(b) LAK cells and colon carcinoma cells were from the same specimen.

(c) LAK cells from the same specimen were tested against two different colon carcinoma targets.

LPMC and normal donor PBMC were tested within one day of isolation for cytotoxicity against colon carcinoma targets. All five LPMC populations tested either before (data not shown) or after enrichment for NK cells (Table 4.2) showed no significant activity against colon carcinoma cells.

In agreement with previous findings (27), the LPMC displayed minimal levels of cytotoxicity against K562 cells even after enrichment for NK cells (3.9% specific lysis at an E:T ratio of 50:1). Five normal PBMC populations with moderately high levels of NK activity against K562 cells (mean specific lysis of 38.7%, E:T ratio 50:1) displayed minimal cytotoxicity against colon carcinoma targets (mean activity 3.5%). Only one of the donors tested caused significant lysis (10.1%) of colon carcinoma cells (Table 4.2). Peripheral blood LAK cells generated from two of these normal donors displayed levels of activity against colon carcinoma targets that were comparable to those of intestinal LAK cells. The cytotoxic activities of freshly isolated LPMC and donor PBMC and paired LAK cell populations against colon carcinoma and K562 targets are shown in Figure 4.1.

4.2 The phenotype of intestinal LAK cells active against colon adenoma cells.

Phenotypic analysis of intestinal LAK cells active against tumour cells from a colonic adenoma with carcinoma in situ showed that they

Table 4.2. The cytotoxicity of freshly isolated LPMC and normal donor PBMC against colon carcinoma cells.

Intestinal disorder	Target cells	
	colon carcinoma	K562
colon carcinoma (autologous)[a]	0.0%[b]	4.1%
colon carcinoma (autologous)	0.4%	4.9%
colon carcinoma (autologous)	0.3%	5.8%
colon carcinoma	0.8%	0.2%
diverticular disease	1.0%	4.4%
Donor PBMC (initials)		
PG	0.2%	52.3%
PH[c]	6.2%	33.9%
PH	0.3%	33.9%
DS	10.1%	34.9%
WA	0.0%	45.4%
JR[c]	1.2%	26.8%
JR	0.0%	26.8%

LPMC isolated from intestines resected for the conditions shown and PBMC from five normal donors were enriched for NK cells and then tested for cytotoxicity against colon carcinoma and K562 cells.

(a) LPMC and colon carcinoma cells were from the same patient.

(b) Percent specific release from target cells at an E:T ratio of 50:1.

(c) The donor PBMC populations indicated were each tested against two different colon carcinoma targets.

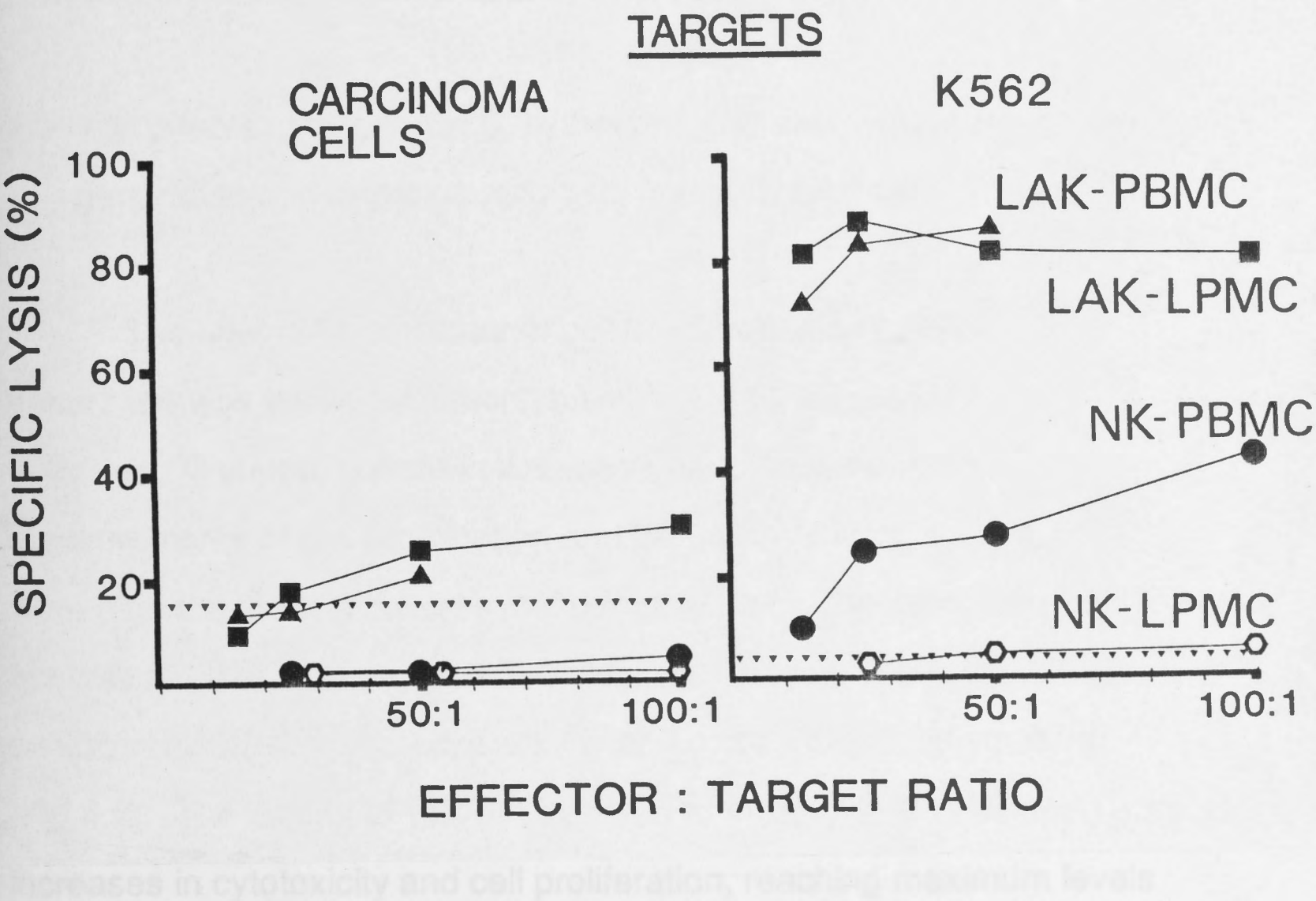

Figure 4.1 The activity of intestinal LAK cells and LPMC against autologous colon carcinoma cells. The cytotoxic activity of intestinal LAK cells generated by culture of LPMC with IL-2 (■) is compared to that of freshly isolated LPMC (O) and also with freshly isolated PBMC (●) and peripheral blood LAK cells (▲) from a normal donor. Cytotoxicity is expressed as percent specific release from colon carcinoma cells at E:T ratios from 12.5 to 100:1.

were $CD2^{+}3^{-}16^{-}$ (Table 4.3). These findings agree with previous phenotypic studies of intestinal LAK cells using K562 targets (27).

4.3 The effects of mitomycin C, hydrocortisone and cyclosporin on the generation and cytotoxic activity of intestinal LAK cells.

The effect of an inhibitor of cell proliferation on LAK cell generation was tested by treating a single LPMC population with mitomycin C prior to culture in IL-2-containing medium. Daily measurements of cell proliferation and LAK activity against K562 cells were made in the test cultures and compared with concurrent control cultures of LPMC treated with IL-2 alone. Mitomycin C-treated LPMC exhibited minimal proliferative activity and cytotoxicity during culture (Fig 4.2). The control LPMC not exposed to mitomycin C developed parallel increases in cytotoxicity and cell proliferation, reaching maximum levels on the fifth and sixth days of culture respectively. The presence of mitomycin-C in the concentrations used did not affect the viability of LPMC for up to 24 h after treatment or the activity of intestinal LAK cells already generated by culture with IL-2 (data not shown).

When hydrocortisone was added to LPMC cultured with IL-2, inhibition of LAK cell generation occurred in all ten specimens treated. The inhibitory concentrations were equal to, or substantially less than, physiological serum levels of cortisone (10^{-6}M) (420). The sensitivity of LPMC populations to hydrocortisone varied widely. For the three

Table 4.3 The phenotype of intestinal LAK cells tested against adenoma cell targets.

Indication for intestinal resection	C control[(a)]	OKT3	OKT11	Leu-11b
colon carcinoma	36.2%[(b)]	32.4%	16.5%	30.4%
colon carcinoma	15.8%	16.8%	3.0%	13.4%
colon carcinoma	22.0%	18.9%	10.0%	17.5%

(a) The cytotoxic activities of intestinal LAK cells treated with the indicated monoclonal antibodies and complement are compared with LAK cells treated with complement alone (C control)

(b) Percent specific release from colon carcinoma cells at an E:T ratio of 50:1.

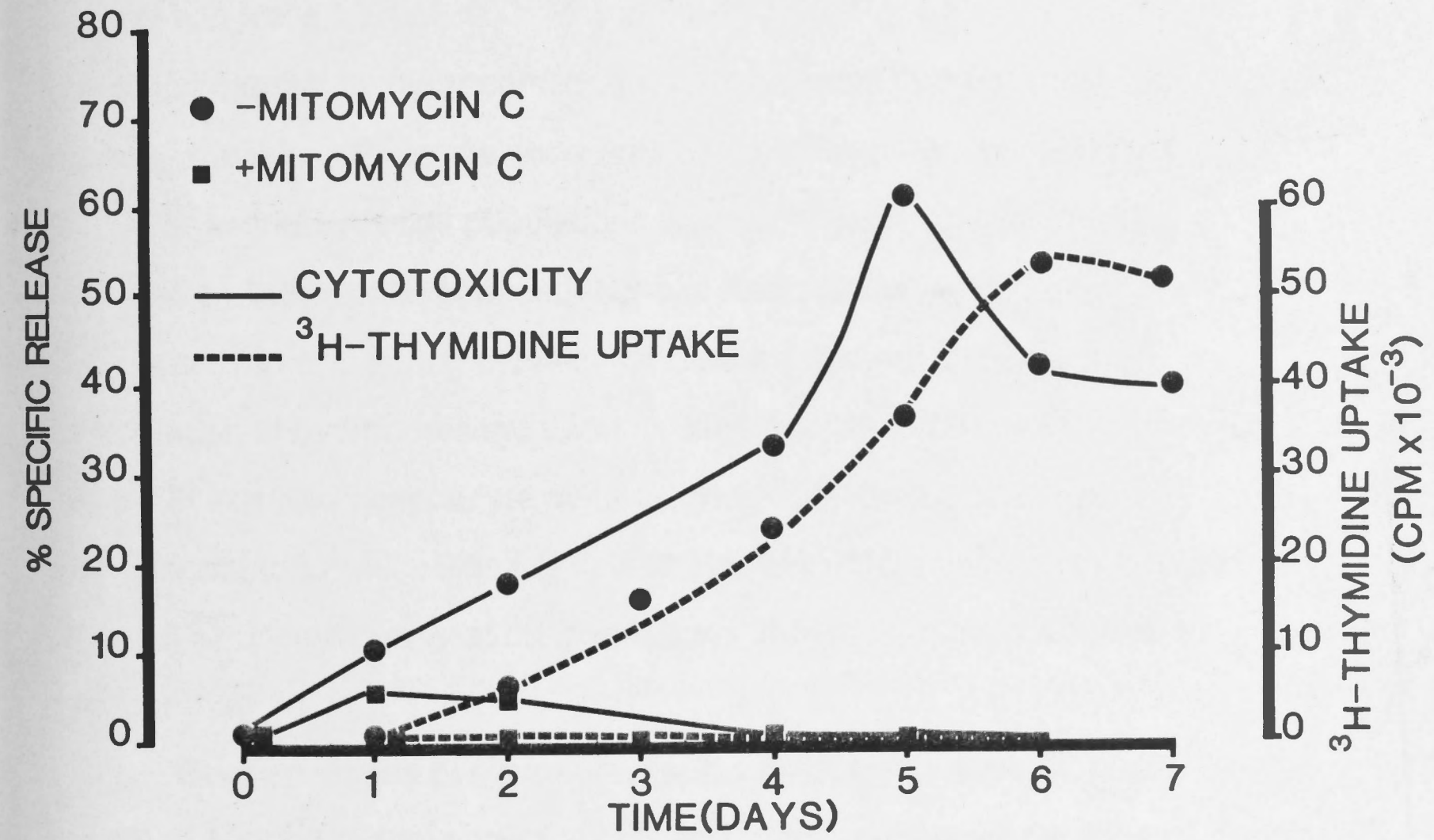

Figure 4.2 The effect of mitomycin C on the generation of intestinal LAK cells. LPMC were cultured in the presence of IL-2 with or without prior treatment with 50 μg/ml mitomycin C. Cell proliferation measured by ^{3}H-thymidine uptake and cytotoxicity against K562 targets (% specific release) were measured serially in the treated and untreated specimens.

specimens with the minimum, median and maximum sensitivities to hydrocortisone, the concentrations causing 50% inhibition of LAK cell generation were 10^{-6} M, 10^{-8} M and 5 x 10^{-9} M (Fig 4.3). The degree of inhibition caused by hydrocortisone was not related to the level of LAK activity that was achieved without treatment. In addition, the activities of two intestinal LAK cell populations against K562 cells were not affected by therapeutic concentrations of hydrocortisone (10^{-5} M) present for 1 h prior to and during cytotoxicity testing (data not shown). The preparation of hydrocortisone used for the LAK cell experiments was also tested in in mixed lymphocyte cultures and PHA-stimulated cultures of unfractionated LPMC. Significant inhibition of LPMC proliferation (greater than 30%) occurred only at concentrations of hydrocortisone equal to or greater than 10^{-5} M.

Concentrations of cyclosporin approximating therapeutic blood levels of 100-400 ng/ml serum (421) had no effect on the generation of intestinal LAK cells (Table 4.4). Addition of the same concentrations to LAK cells immediately before the cytotoxicity assay had no effect upon their activity towards K562 cells (data not shown). Similar concentrations of cyclosporin blocked LPMC proliferation in response to alloantigens or PHA by more than 75%.

4.4 The effect of interferon on intestinal LAK cell activity.

Four LPMC populations with culture-generated LAK cell activity

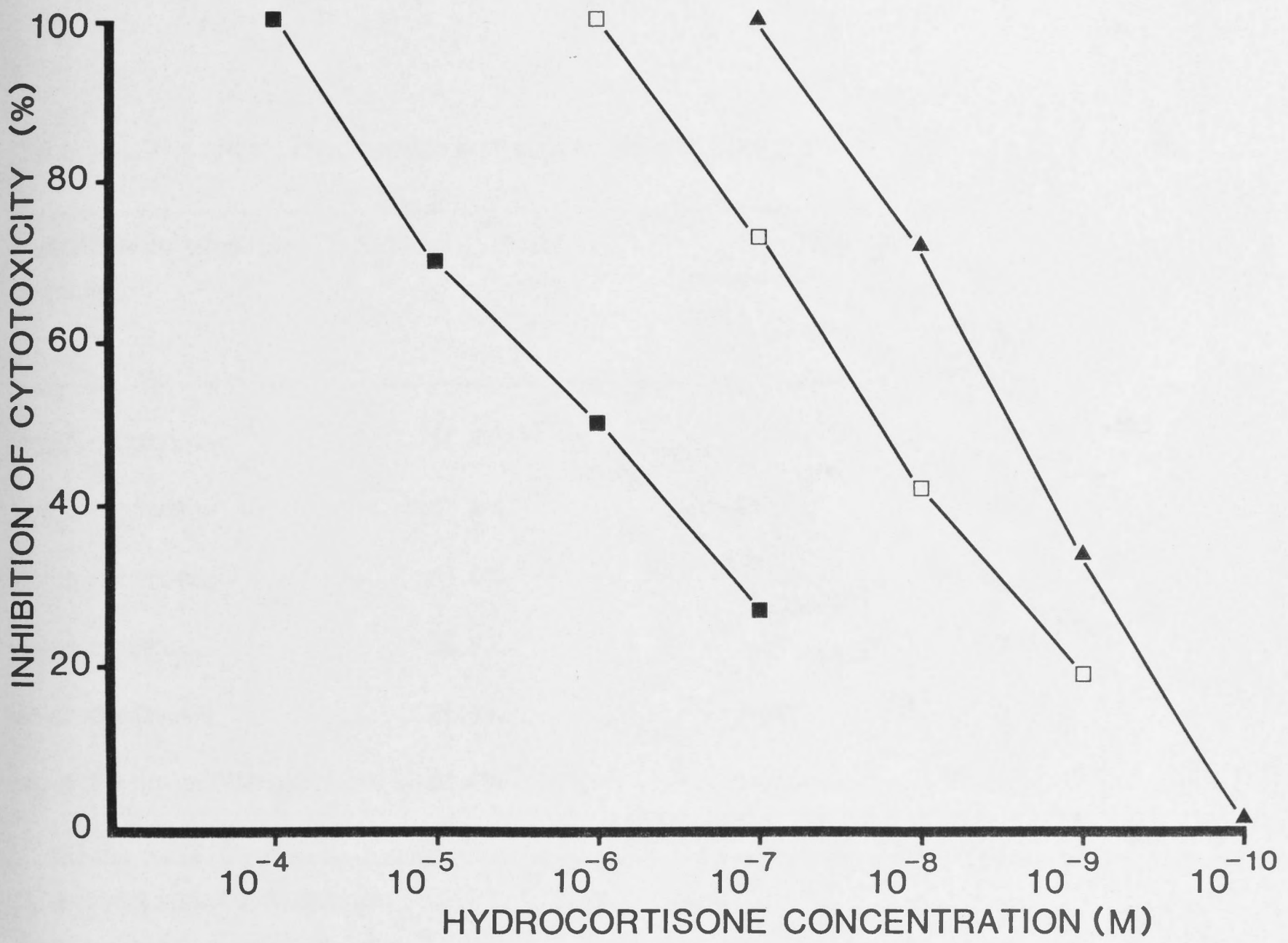

Figure 4.3 The effect of hydrocortisone on the generation of intestinal LAK cells. Hydrocortisone in the concentrations shown was added at the initiation of cultures of LPMC with IL-2. After six days' culture, the cytotoxicity of treated and control LPMC was tested against K562 cells. The effect of hydrocortisone was expressed as the percent inhibition of LAK cell activity after treatment compared with control cultures. Of the 10 populations tested, those with the minimum (■), median (□) and maximum (▲) sensitivity to inhibition by hydrocortisone are shown.

Table 4.4 The effect of cyclosporin on the generation of intestinal LAK cells.

Indication for intestinal resection	Control (IL-2 alone)	Cyclosporin + IL-2	
		0.1 µg/ml	0.3 µg/ml
colon carcinoma	15.8%[a]	20.3%	15.4%
colon carcinoma	61.9%	64.5%	67.6%
colon carcinoma	61.4%	62.8%	61.0%
colon carcinoma	35.5%	- [b]	37.7%
colon carcinoma	26.9%	23.5%	23.8%
adhesive bowel obstuction	61.4%	57.5%	49.7%

(a) percent specific release from K562 cells at an E:T ratio of 50:1.

(b) experiment not performed.

were treated with IFN in concentrations of 125 U/ml and 250 U/ml. Doses of IFN which cause maximum stimulation of peripheral blood NK activity (87,88) produced only slight increases in LAK cell activity (Table 4.5).

IV. Discussion

This study shows that intestinal LAK cells lyse colon carcinoma cells that are resistant to lysis by freshly isolated LPMC. Similarly, peripheral blood LAK cells but not unstimulated PBMC can lyse colon tumour cells, a finding consistent with earlier observations in which colon cancer and other fresh tumour cells were used as targets (79). Earlier studies reported lysis of colon carcinoma cells by autologous lymphocytes from peripheral blood, mesenteric lymph nodes and the tumour itself and attributed this activity to cytotoxic T cells (206). In the present study, it is unlikely that the activity observed was due to specific cytotoxic T cells since the lysis was not MHC-restricted and the phenotype of intestinal LAK cells was not that of cytotoxic T cells or NK cells. Moreover, the LAK precursor cell populations were selected from the 1.063 g/ml Percoll interface which is depleted of T cells (25).

The ability of intestinal LAK cells to kill fresh colon cancer cells in vitro lends support to their proposed role as an antineoplastic cell in vivo. It could be argued that the lysis of colon carcinoma cells by LAK cells reflects a high level of nonspecific cytotoxicity which has little particular relevance to the pathogenesis of colon cancer. Indeed, the

Table 4.5 The effect of interferon (IFN) on intestinal LAK cell activity.

Indication for intestinal resection	Control (no IFN)	IFN added	
		125 U/ml	250 U/ml
colon carcinoma	67.8%[(a)]	78.9%	75.3%
colon carcinoma	61.0%	60.8%	64.0%
colon carcinoma	36.0%	34.5%	39.6%
colon carcinoma	12.2%	13.2%	14.8%

(a) percent specific release from K562 targets at an E:T ratio of 50:1.

possibility that LAK cells lyse nonmalignant cells supports this argument, although there is disagreement about the extent to which this occurs (288,289). However, the recent observation that intestinal LAK cells do not lyse normal colonic epithelial cells suggests that these cells display selective reactivity against malignant epithelial cells (422).

The phenotypic differences between $CD2^+3^-16^-$ intestinal LAK cells cytotoxic towards K562 (27) or colon carcinoma targets and peripheral blood LAK cells are probably accounted for by the limited range of LAK precursor cell types available in the intestinal mucosa. The major peripheral blood LAK effector cells are NK cell-derived ($CD2^+3^-16^+$Leu-19^+) or T cell-derived ($CD2^+3^+16^-$) but a small population with a phenotype similar to the intestinal LAK cell ($CD2^+3^-16^-$Leu-19^+) has been reported (38,39). The lack of NK or T cell-associated markers on intestinal LAK cells implies therefore, that the intestinal LAK precursor cell differs from its counterparts in the peripheral blood. There is also phenotypic evidence from this laboratory (27) and from others (321) reporting the existence of a $CD2^+$ intestinal LAK precursor cell which lacks markers found on NK cells (CD16) or T cells (CD3).

Evidence that LAK cells may exist and function <u>in vivo</u> comes from adoptive transfer studies in both man and experimental animals where culture-generated LAK cells have significant anti-tumour effects (111,423). Moreover, lymphocytes with cytotoxic properties similar to those of LAK cells have been isolated from the antigen-stimulated murine intestinal epithelium (343); after IL-2 administration to mice (346) and in

man from the joint fluid in active rheumatoid arthritis (347) and in the blood following bone marrow transplantation (65). Although no direct evidence of LAK cells has been found in freshly isolated LPMC or tumour-infiltrating lymphocyte populations, the potentially suppressive effect of the isolation procedure may mask their presence (27,352 354,356,358). The lack of IL-2 in the medium during the long period of isolation is also potentially suppressive as the activity of LAK cells declines rapidly in the absence of IL-2 (Chapter 3). The apparent lack of LAK cells in the intestine is unlikely to be a consequence of low IL-2 levels in the mucosa, as biologically significant amounts of this lymphokine are released by mitogen-stimulated LPMC irrespective of the underlying intestinal disorder (412,413,424 and Chapter 7).

Kinetic analysis of LAK activity and cell proliferation in the Mitomycin C experiments confirms previous findings that proliferation of cells is integral to the generation of LAK activity (354). The suppression of intestinal LAK cell generation by hydrocortisone is similar to that reported for peripheral blood LAK cells (295,425) and the concentrations inhibitory for LAK cell generation (10^{-6} M or lower) contrast with the substantially larger concentrations (10^{-5} M or higher) needed to inhibit IL-2 production and resulting T cell activation in alloantigen or mitogen-stimulated cultures (295,426,427). In the presence of 10^{-5} M hydrocortisone however, the response of previously activated T cells to exogenous IL-2 is unaffected (427). Thus, the inhibition of LAK cell generation by lower concentrations of hydrocortisone suggests that there

may be differences between the mechanisms of activation of LAK precursor cells and T cells by IL-2. On the other hand, the resistance of LAK cell generation to cyclosporin suggests that this process has features in common with the IL-2-induced proliferation of T cell blasts (427) and activation of NK cells (428), both of which are also unaffected by similar concentrations of cyclosporin.

The reason for the minimal effect of IFN on LAK activity may be that the cells are already maximally activated by IL-2 alone, or in combination with other factors produced during culture. A recent study supports this view by showing that LAK cell generation is dependent not only on exogenous IL-2 but also upon endogenous production of IFN in culture (69). In accordance with studies on peripheral blood LAK cells (295), the cytotoxicity of intestinal LAK effector cells was unaffected by mitomycin C and therapeutic concentrations of hydrocortisone and cyclosporin.

In conclusion, this study has demonstrated that NK cell-resistant colon carcinoma cells are lysed by intestinal LAK cells. The generation of intestinal LAK cells appears to be controlled by mechanisms similar to those operating for peripheral blood LAK cells, as shown by the inhibitory effect of mitomycin C and hydrocortisone but not cyclosporin in induction cultures. The ability of intestinal LAK cells to lyse colon carcinoma cells supports their postulated role as an important component of mucosal defence against the development of colon carcinoma.

CHAPTER 5.

Intestinal lymphokine-activated killer cells in inflammatory bowel disease.

I. Introduction

Efforts to identify an underlying immunological mechanism for Inflammatory Bowel Disease (IBD) have focused recently on the immunoregulatory and cytotoxic functions of intestinal lymphocytes. Substantial evidence indicates that abnormalities in immunoregulation by T cells from the intestinal mucosa may be important in predisposing towards IBD (365,430,431). By comparison, little is known about the involvement of cytotoxic lymphocytes in the pathogenesis of IBD (336, 350,353,356). Early reports showing that peripheral blood mononuclear cells (PBMC) from patients with IBD lysed colonic epithelial cells stimulated interest in natural killer (NK) cells as a potential cause of mucosal damage in IBD (432,433). However the importance of NK cells is now in doubt, as most studies of intestinal lymphocytes from IBD mucosa report minimal levels of NK activity (336,350,353). Moreover, even in studies where significant mucosal NK activity was reported in IBD, the levels were comparable to those assayed in histologically normal mucosa (356). Interpretation of these data is also limited by the use of K562 target cells, which may not detect the presence of cytotoxicity directed

against epithelial cells and other cell types present in the intestinal mucosa.

Previous studies of intestinal mucosal lymphocytes have reported the generation of lymphokine-activated killer (LAK) cells which are potentially important nonspecific cytotoxic cells in the intestinal mucosa (27,197,321,354). Intestinal LAK cells were generated by culture of LPMC with interleukin 2 (IL-2) and displayed levels of cytotoxicity markedly higher than the minimal spontaneous NK activity of freshly isolated LPMC. Intestinal LAK cells lysed not only NK-sensitive and resistant tumour cell lines (27,354) but also freshly isolated colon carcinoma cells which were NK cell-resistant (197).

In the present study the role of intestinal LAK cells in the pathogenesis of IBD was investigated. The aims of this study were to determine whether intestinal lymphocytes isolated from mucosae affected by IBD possessed IL-2-inducible LAK activity and to test this LAK activity in IBD against target cells isolated from the intestinal mucosa. The LAK cell populations generated in IBD were examined for cytotoxic activity against epithelial tumour cells and fibroblasts freshly isolated from the intestinal mucosa and were compared to LAK cells from histologically normal mucosae. The ability of intestinal lymphocytes from IBD to display LAK activity against target cells of intestinal origin suggests that these cells may contribute to mucosal injury in IBD.

II. Materials and Methods

5.1 Patients and specimens.

Eighteen specimens of mucosa were taken from surgically resected intestine from patients with IBD. There were 11 specimens of ileum and three of colon obtained from 13 patients with Crohn's disease and four specimens (three colon and one ileum) from three patients with ulcerative colitis. At the time of resection, all patients with Crohn's disease and one with ulcerative colitis were receiving corticosteroids; additional therapy with azathioprine was being administered in four cases of Crohn's disease and one of ulcerative colitis. Histological examination of the 18 mucosal specimens with IBD demonstrated active inflammation in all cases. The diagnoses of Crohn's disease and ulcerative colitis were based on standard clinical, radiological and histological criteria.

Specimens of histologically normal intestinal mucosa were also obtained from patients undergoing surgical resection for colon carcinoma (12 cases), diverticular disease (seven cases) and sigmoid volvulus (one case). Tissue from which tumour cells and fibroblasts were isolated was obtained from three colon carcinomas, one colonic adenoma and the normal intestinal mucosa of four patients undergoing surgical resection for colon carcinoma.

5.2 Preparation of fibroblast target cells from intestinal mucosa.

Intestinal fibroblasts were obtained by disaggregation of histologically normal mucosa resected for carcinoma or nonmalignant conditions. The mucosa was scraped from the muscularis, minced finely and digested as described for colon carcinoma tissue. The digest was centrifuged at 50g for 3 min and the supernatant was then washed and cultured as described for colon carcinoma cells. Fibroblasts formed an adherent continuous monolayer by four weeks in culture. There was no contamination of cultures by mononuclear or intestinal epithelial cells as determined by morphological and surface marker studies (data not shown).

When required for use as target cells, adherent fibroblast monolayers were resuspended by exposure for 15 min at 37^{o}C to calcium and magnesium-free Hank's balanced salt solution containing 0.5mM EDTA and 0.2% trypsin. The detached cells were washed twice and resuspended in medium supplemented 5% FBS for labelling with ^{51}Cr.

The mean spontaneous release expressed as a percentage of maximum release was 16% for fibroblasts. Specific release was considered to represent significant cytotoxic activity when it exceeded a cut off value equal to the mean plus three standard deviations of the spontaneous release controls. This cut off value for significant cytotoxicity for fibroblast target cells was 5.4%.

Details of LPMC and PBMC isolation, NK cell enrichment, the cytotoxicity assay and statistical analysis are given in Chapter 3.

Lymphokine-activated killer cells were generated using 250 U/ml human recombinant IL-2 as described in Chapter 3.

III. Results

5.1 Spontaneous cytotoxicity of LPMC tested against K562 cells.

Eighteen LPMC populations isolated from IBD mucosae and twenty populations from uninflamed mucosa were enriched for NK cells and tested for spontaneous cytotoxicity against K562 cells. The LPMC populations from IBD displayed very low or insignificant levels of NK activity (Fig 5.1). This contrasted with the much higher levels of NK activity exhibited by enriched PBMC from normal donors tested in the same assay ($p < 0.01$). As previously reported (11), the spontaneous NK activity of LPMC populations from histologically normal mucosa was also very low (mean specific releases of 1.1% and 0.6% for colorectal carcinoma and non-malignant conditions respectively). These values were not significantly different from the IBD specimens.

5.2 LAK activity of LPMC tested against K562 cells.

Intestinal LAK activity was generated by culture of LPMC in IL-2-containing medium and was assayed against K562 targets. The marked difference between the spontaneous NK activity and

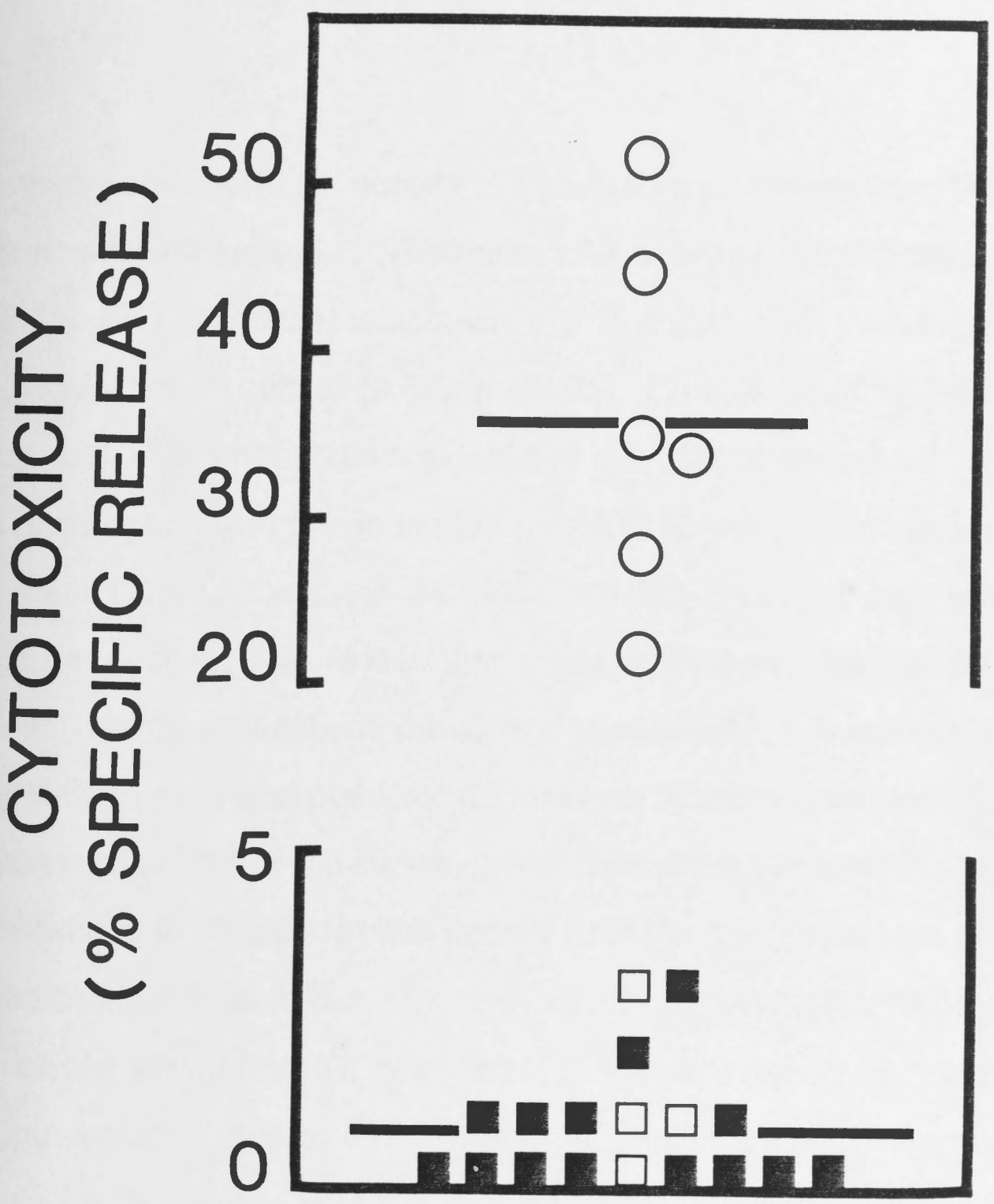

Figure 5.1 The spontaneous NK activity of LPMC isolated from patients with Crohn's disease (■) and chronic ulcerative colitis (□). Shown for comparison are the NK activities of PBMC (O) from six normal subjects assayed under identical conditions. NK activity is expressed as % specific release from K562 cells at an E:T ratio of 50:1 and mean NK activities for the two groups are indicated by horizontal bars. The mean NK activity of PBMC was significantly greater than that of LPMC ($p < 0.01$).

culture-generated LAK activity of 11 LPMC populations from IBD is illlustrated in Figure 5.2. The mean LAK activity of LPMC from IBD was 28.3% (E:T ratio 50:1) which was significantly higher than their NK activity prior to culture (0.6%; $p < 0.01$). Cultures of LPMC in medium without IL-2 did not acquire significant cytotoxicity (mean activity 0.2%). In accordance with previous studies of LPMC from histologically normal mucosa (27), LAK activity of LPMC from IBD increased progressively during culture reaching peak levels after six days (data not shown). While nine of eleven LPMC populations developed LAK activity, two specimens (one ulcerative colitis and one Crohn's disease) failed to gain significant cytotoxicity. These two cases did not differ from the remaining specimens in clinical disease activity or in the dosage or type of medication administered. The LAK activity of enriched LPMC from mucosae affected by IBD was not significantly different from that of LPMC populations from colon carcinoma or nonmalignant disorders (mean LAK activities 34.8% and 30.3% respectively).

5.3 The NK and LAK activity of LPMC tested against tumour cells and fibroblasts from the intestine.

In view of the high levels of LAK activity against K562 cells, LPMC from five cases of Crohn's disease were also tested for cytotoxic activity against target cells of intestinal origin. Freshly isolated LPMC and the corresponding LAK cell populations were assayed for NK and LAK activity

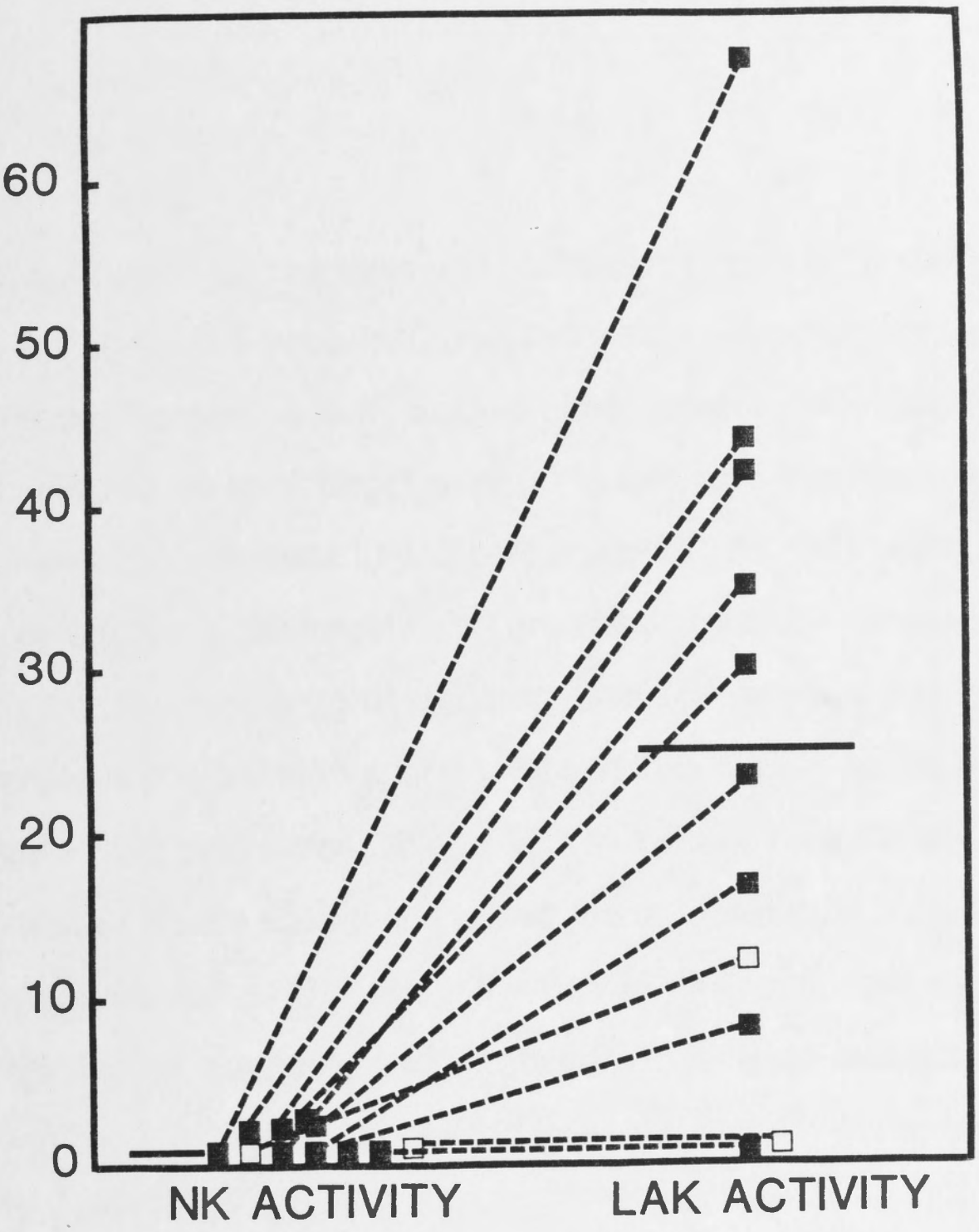

Figure 5.2 The lymphokine-activated killer (LAK) cell activity of LPMC isolated from intestines resected for Crohn's disease (■) and ulcerative colitis (□) after culture in medium supplemented with IL-2. For each specimen the maximum LAK activity developed against K562 cells is compared with the spontaneous NK activity present before culture. Cytotoxic activity is expressed as % specific release at an E:T ratio of 50:1 and the mean activities before and after culture with IL-2 are indicated by horizontal bars. The mean LAK activity for the 11 specimens was significantly greater than their spontaneous NK activity ($p < 0.01$).

against both colon tumour cells and fibroblasts (Table 5.1). Before culture with IL-2, none of the five LPMC populations displayed significant spontaneous cytotoxic activity against either tumour or fibroblast targets (mean activities for each target were 0.7% and 1.0% respectively). In four of the five Crohn's disease LPMC preparations, LAK cells with high levels of activity against K562 targets also mediated significant cytotoxicity towards target cells disaggregated from colon carcinomas and an adenomatous polyp (mean specific release 19.9%). Intestinal mucosal fibroblasts were also susceptible to lysis by LAK cell populations from all four Crohn's disease specimens tested (mean specific lysis 29.6%). Further studies using PBMC from five normal control donors showed that colon carcinoma, adenoma and fibroblast targets were resistant to lysis by NK cell populations which caused an average of 37.8% specific lysis of K562 targets (Table 5.1).

Lymphokine-activated killer cells from histologically normal specimens of mucosa resected for colon carcinoma served as disease controls. These intestinal LAK cell populations also lysed colon carcinoma and fibroblast targets. As presented fully in chapter 4, colon carcinoma cells were lysed by LAK cells from eight mucosal specimens resected for colon carcinoma (mean specific lysis 22.7%). Three of these populations lysed intestinal fibroblasts with a mean specific lysis of 40.4% (Table 5.1). These results indicate that LAK cells from IBD specimens lysed NK-resistant intestinal tumour cells and fibroblasts. This finding however, was not confined to IBD, as LAK cells from uninflamed mucosa

Table 5.1 The NK and LAK activity of LPMC assayed against tumour cells and fibroblasts from the intestinal mucosa and K562 cells.

Intestinal disorder	Target cells					
	colon carcinoma/adenoma		fibroblast		K562	
	NK [a]	LAK [a]	NK	LAK	NK	LAK
Crohn's disease[b]	0.2	52.8[c]	0.4	41.1	0.2	70.2
	1.2	22.0	-[d]	-	0.0	70.2
Crohn's Disease	2.9	7.0	1.3	30.3	0.7	62.9
Crohn's Disease	0.0	10.1[e]	0.4	19.8	0.8	36.4
Crohn's Disease	0.9	15.1[e]	2.2	27.0	1.2	43.3
Crohn's Disease	0.3	12.1	0.9	-	0.8	19.8
	NK	LAK	NK	LAK	NK	LAK
colon carcinoma	0.0	12.3	4.4	33.6	4.4	41.9
colon carcinoma	0.7	48.4	2.0	58.9	6.3	75.8
colon carcinoma	-	35.6	0.7	28.8	-	51.8
Donor PBMC (initials)						
	NK		NK		NK	
PH	6.5		5.2		30.2	
PG	0.7		4.2		52.4	
JR	1.2		2.3		26.9	
DS	10.0		-		34.8	
WA	0.0		-		44.9	

(a) For each LPMC specimen both the NK and LAK activities against each target are listed. Only the NK activity of PBMC was studied.

(b) LPMC from this specimen was assayed against two different colon carcinoma targets.

(c) Cytotoxic activity is expressed as % specific release from target cells at an E:T ratio of 50:1.

(d) Experiment not performed

(e) Adenoma cell targets.

resected for colon carcinoma also lysed the same targets.

IV. Discussion

The present investigation of intestinal NK and LAK cells addresses recent controversy concerning the role of nonspecific cytotoxic cells in neoplastic and inflammatory diseases of the human intestine (400,434). In agreement with previous reports using comparable assay conditions, the spontaneous NK activity of LPMC from IBD was found to be minimal and did not differ from the similarly low cytotoxic activity found in histologically normal intestinal mucosa (350,353,336). Moreover, normal and neoplastic target cells from the intestine are insensitive to lysis by freshly isolated LPMC. These findings suggest that NK cell-mediated cytotoxicity may not be important in the pathogenesis of mucosal injury in IBD.

On the other hand, both the present and previous studies have demonstrated the presence of IL-2-inducible LAK cell precursors in the histologically normal or inflamed intestinal mucosa (27,321,354). The chronic inflammatory lesion of IBD is characterized by infiltration of the mucosa with T cells which may release cytokines such as IL-2 into the mucosal microenviroment as part of local immune events. Thus, appropriate and sustained growth factor production may be present for the generation of LAK cells in the inflamed intestinal mucosa in vivo. Indeed, intestinal lymphocytes with cytotoxic properties similar to LAK cells have been isolated from immunized animals following oral challenge with

large doses of antigen (346). Furthermore, LAK cells are present in synovial fluid extracted from inflamed joints of patients with active rheumatoid arthritis (347).

To date, few of the reported studies investigating the role of intestinal LAK cells has used target cells that are relevant to inflammation of the intestinal mucosa (197,336,350,354). In order to establish the pathogenetic significance of intestinal LAK cells in IBD, it is essential to demonstrate that these effector cells are cytolytic towards other cells derived from the intestinal mucosa. The study reported here demonstrates that neoplastic epithelial cells and normal fibroblasts freshly obtained from intestinal mucosa are sensitive to the cytotoxic action of LAK cells from IBD mucosae. Such activity is not exclusive to IBD as LAK cells from histologically normal mucosa also displayed comparable levels of cytotoxicity against intestinal target cells. Nevertheless, the presence of mucosal LAK cell precursors in IBD and the ability of intestinal LAK cells generated in vitro to lyse biologically relevant intestinal target cells suggest a role for LAK cells in producing mucosal injury in the inflamed intestinal mucosa.

At present there is no direct evidence for the existence of intestinal LAK cells in vivo in IBD. No specific phenotypic markers able to detect LAK cells have been reported, although intestinal LAK cells were shown to be $CD2^{+}3^{-}16^{-}$Leu-7^{-}(27). As reported in this and other studies, freshly isolated LPMC show little evidence of spontaneous LAK activity (27,354). This observation may not be unexpected however, as both

suppressive and cytotoxic factors are released during isolation of LPMC from the intestine (358). Another contributing factor may be the lack of detectable IL-2 in the digest during the period of isolation, as intestinal LAK cell activity declines rapidly in the absence of IL-2 (Chapter 3). A complementary approach to examining the question of LAK cell generation in vivo is to examine the capacity of LPMC to produce IL-2 in vitro. Populations of LPMC from patients with IBD are capable of releasing IL-2 when stimulated by phytohaemagglutinin (414,435) although reduced synthesis was noted when the stimulating factor was phorbyl-myristic acetate (414).

In conclusion, LAK cells generated from LPMC from intestinal mucosae affected by IBD are capable of lysing normal and neoplastic target cells freshly isolated from the intestinal mucosa. The presence of precursors of LAK cells in the intestinal lamina propria and their ability to lyse biologically relevant intestinal cells in vitro combined with the likely production of IL-2 in the microenviroment of the inflamed mucosa suggest that LAK cells may contribute to intestinal damage in IBD.

CHAPTER 6.

Antibody-dependent cellular cytotoxicity of lamina propria mononuclear cells from the human intestinal mucosa: the effect of interleukin 2 and characterization of the effector cells.

I. Introduction

Although the cytotoxicity of intestinal lymphocytes has been studied extensively, little is known about their role in mucosal defence mechanisms or in the pathogenesis of intestinal diseases (400,434). Recent studies of nonspecific cytotoxicity in the intestinal mucosa have concentrated on natural killer (NK) cell activity (27,354,356,321), antibody-dependent cellular cytotoxicity (ADCC)(350,352,363,364) and lymphokine-activated killer (LAK) cell activity (27,197,321,354). Lymphocytes mediating ADCC are classified separately from NK cells as killer (K) cells (53), although recent evidence indicates that the majority of K cells in the peripheral blood may be identical to, or overlap substantially with, the lymphocyte population displaying NK activity (50,53). In contrast to the nonspecific cytotoxicity of NK and LAK cells, ADCC is directed specifically against targets by the interaction of Fc receptors on K cells with antibody directed against a target cell surface antigen (53). Through this mechanism, K cells may participate not only in the protection of the normal intestinal mucosa but also in the

pathogenesis of intestinal diseases. The reported ADCC of intestinal lymphocytes against enteric bacterial and protozoan pathogens suggests that K cells may protect the intestine against microbial invasion (47,48,213). Furthermore, the antineoplastic properties of K cells (53) may be important in preventing the development of neoplasia in the intestinal mucosa.

Despite the potential importance of mucosal K cells, several studies have shown that human intestinal lymphocytes display minimal levels of spontaneous ADCC against nucleated cell line targets (350,352,363,364). Similar results reported for intestinal NK cell activity suggest that cells of the NK/K type are infrequent in the human intestinal mucosa (27,354). Previous studies of human lamina propria mononuclear cells (LPMC) reported that low levels of spontaneous cytotoxicity against K562 cells could be enhanced markedly by culture of intestinal lymphocytes in medium containing interleukin 2 (IL-2) (27,321,354). The cytotoxic cells induced by IL-2 were characterized as intestinal LAK cells (27) because of their ability to lyse NK-resistant tumour cell targets and also by their lack of expression of the NK/K cell-associated surface markers Leu-7 (394) and CD16 (436). Peripheral blood-derived LAK cells differ from intestinal LAK cells in that the majority arise from NK cells and express the CD16 surface antigen (284).

The objectives of this study were to investigate the effect of IL-2 on the ADCC of lamina propria mononuclear cells (LPMC) isolated from histologically normal human intestinal mucosa and to identify the

intestinal cells responsible for ADCC. The ADCC of paired LPMC populations was therefore measured and compared immediately upon isolation and after culture in IL-2-containing medium. It was found that freshly isolated LPMC had minimal spontaneous ADCC, but IL-2-stimulated LPMC mediated substantial levels of ADCC. Phenotypic analysis showed that the IL-2-induced ADCC of LPMC was mediated by both $CD16^-$ and $CD16^+$ LAK effector cell populations. These results suggest that intestinal LAK cells generated from IL-2-stimulated LPMC mediate high levels of ADCC which have potentially important functions in the normal and diseased intestinal mucosa.

II. Materials and Methods

6.1 Mucosal specimens.

Lamina propria mononuclear cells (LPMC) were isolated from samples of histologically normal colonic mucosa resected for colonic carcinoma (eight patients); diverticular disease (two patients); sigmoid volvulus (one patient) and ileocaecal lipoma (one patient).

6.2 Preparation of target cells.

Target cells for the cytotoxicity assay were from the K562, P815 and HL60 cell lines. One million cells from each cell line were incubated

for 1 h in medium containing 50 μCi of sodium ^{51}chromate and 10% FBS. The target cells were then washed 4 times prior to cytotoxicity testing. Targets for measurement of ADCC were P815 cells labelled with anti-P815 antibody (P815-IgG targets). Anti-P815 antibody was prepared by immunization of rabbits with P815 cells in complete Freund's adjuvant. Multiple serum specimens were collected and the IgG fraction prepared by ammonium sulphate precipitation. The specificity of the antibody preparation was confirmed by demonstration of antibody and complement-mediated cytotoxicity of P815 cells but none of the other tumour cell lines used in this study. To prepare P815-IgG targets, 5 x 10^5 ^{51}Cr-labelled P815 cells were incubated with an optimal dilution of anti-P815 antibody in medium (I/20) for 20 min at room temperature and were then washed three times before cytotoxicity testing.

The spontaneous release of ^{51}Cr from each target cell expressed as a percentage of maximum release was as follows : P815 (14%); P815-IgG (13%) and HL60 (13%). Specific release was considered to represent significant cytotoxic activity when it exceeded a cut off value equal to the mean plus three times the standard deviation of spontaneous release controls. Mean cut off values for the target cells were 4.4% (P815); 4.2% (P815-IgG) and 3.0% (HL60).

6.3 Cold target inhibition experiments.

Two LPMC populations with IL-2-induced LAK activity were tested

against ^{51}Cr-labelled P815-IgG target cells in the presence of unlabelled (cold) P815, P815-IgG, K562 or HL60 targets. Varying numbers of cold targets of each type were combined with 5 x 10^3 ^{51}Cr-labelled P815-IgG cells and 1.25 x 10^5 LPMC in 0.2 ml of medium in triplicate microculture wells to produce competitor to labelled target ratios from 0.5:1 to 20:1. The cytotoxic activity of LAK cells towards ^{51}Cr-labelled P815, P815-IgG, HL60 and K562 cells in the absence of cold target cells served as controls. After incubation for 4 h at 37^{o}C in an atmosphere of 5% CO_2 in air, the cytotoxic activity of test and control wells was calculated as described in Chapter 3. The effect of cold target cells at each competitor to target cell ratio was expressed as the percent inhibition of the lysis of ^{51}Cr-labelled P815-IgG targets by LPMC in control wells.

6.4 Phenotyping of LPMC by antibody and complement-mediated lysis.

The surface phenotype of LPMC populations displaying IL-2-induced LAK activity towards P815-IgG and K562 targets was determined using antibody and complement-mediated lysis. The monoclonal antibodies used were OKT3 and OKT11 (Ortho Diagnostic Inc., Raritan, NJ) and anti-Leu 11b (Becton Dickinson, Mountain View, Ca). Ten µL of each antibody diluted 1 in 5 in medium was added to 2.5 x 10^5 LPMC in 30µL of medium with 1% FBS in triplicate round bottom microculture wells. After incubation at room temperature for 7 min, 10µL of complement (Rabbit MA, Cedar Lane Laboratories Ltd., Ontario, Canada) was

added to each well and incubated at 37°C for 60 min in an atmosphere of 5% CO_2 in air. In control wells, LPMC were treated with antibody, medium or complement alone. The treated LPMC were then washed three times in the microculture plates and resuspended in individual wells in 0.1 ml of medium with 5% FBS for cytotoxicity testing. The final 1 in 20 dilutions of OKT3 and OKT11 used were shown by fluorescent antibody analysis to produce maximum complement-mediated lysis of the relevant LPMC subpopulation. A 1 in 20 dilution of anti-Leu 11b resulted in 90% or greater reduction in the NK activity and ADCC of normal control PBMC (data not shown).

Details of LPMC and PBMC isolation, NK cell enrichment, the cytotoxicity assay and statistical analysis are given in Chapter 3. Lymphokine-activated killer cells were generated using 250 U/ml human recombinant IL-2 as described in Chapter 3.

III. Results

6.1 The ADCC of freshly isolated lamina propria and peripheral blood mononuclear cells.

The mean spontaneous ADCC of seven freshly isolated LPMC populations (four with colon carcinoma and three with nonmalignant conditions) was 2.0% when tested at an E:T ratio of 50:1 (Fig. 6.1). Simultaneous assay for NK activity against K562 targets showed a

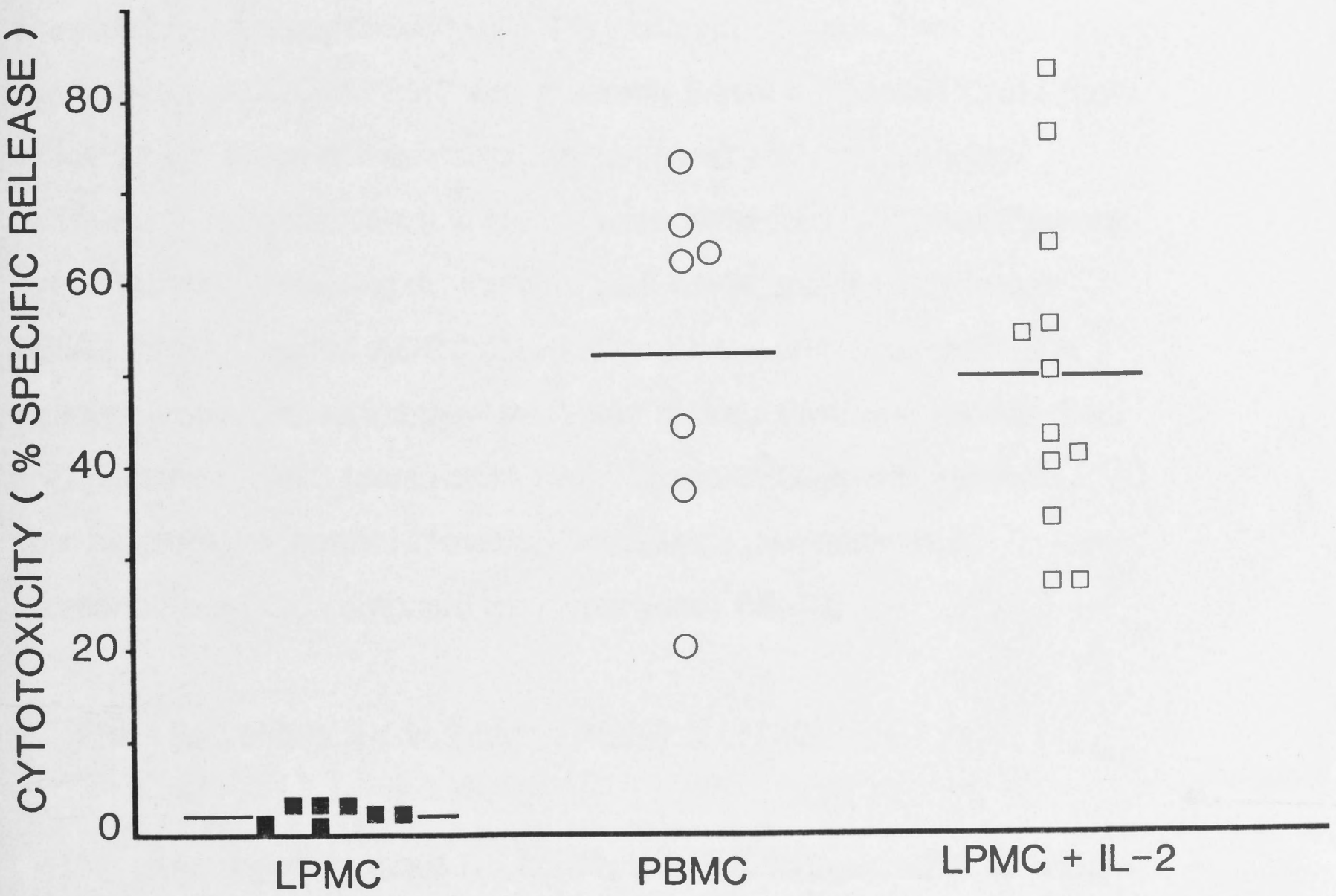

Figure 6.1 The antibody-dependent cellular cytotoxicity (ADCC) of freshly isolated LPMC (■); PBMC from normal donors (O); and LPMC after culture in IL-2-containing medium (□). The horizontal bars shown indicate the mean level of ADCC for each group. ADCC was assayed against P815-IgG targets at an E:T ratio of 50:1 and is expressed as % specific release. The ADCC of freshly isolated LPMC was significantly less than that of PBMC ($p < 0.01$) and intestinal LAK cells ($p < 0.01$).

similarly low mean cytotoxicity of 1.1% (data not shown). The spontaneous ADCC of LPMC was markedly less than the ADCC of PBMC isolated from seven normal donors (mean activity 52.3%; $p< 0.01$). Incubation of LPMC for 24 h in medium supplemented with 3% AB serum before cytotoxicity testing did not alter their ADCC significantly (mean activity 2.5%). The low ADCC of LPMC was not a consequence of the isolation procedure, as identical treatment of two PBMC populations did not affect their ADCC (data not shown). These findings indicate that LPMC from histologically normal intestinal mucosa possess minimal spontaneous ADCC compared to normal donor PBMC.

6.2 The effect of interleukin 2 on the ADCC of LPMC.

As the spontaneous NK activity of LPMC is boosted by incubation with IL-2 (27,321,354), LPMC were cultured with IL-2 to determine if ADCC could be increased. Twelve LPMC populations (including the seven studied in the previous section) were tested for ADCC after culture for up to eight days in the presence of IL-2 (Fig. 6.1). The cultured LPMC populations displayed high levels of activity (mean ADCC 49.2%) which were comparable to normal peripheral blood ADCC and significantly greater than the spontaneous ADCC of LPMC ($p< 0.01$). The ADCC of LPMC increased during culture to a maximum at six days which is similar to the kinetics described in Chapter 3 for intestinal LAK cells. Assay of LPMC cultured for similar periods in medium without IL-2 showed no

spontaneous increase in ADCC (mean activity 1.2%). In agreement with previous results (27,354), the 12 LPMC specimens cultured with IL-2 also developed high levels of cytotoxic activity against K562 targets.

The spontaneous and IL-2-induced ADCC of a representative LPMC population measured over a range of E:T ratios is shown in Figure 6.2. At all E:T ratios up to and including 200:1, the spontaneous ADCC of the LPMC specimen was substantially less than that induced by culture of the same population with IL-2. Untreated PBMC from a normal control subject showed greater spontaneous ADCC than cultured LPMC at lower E:T ratios but similar levels of activity at higher ratios.

6.3 Cold target inhibition of the ADCC mediated by intestinal LAK cells.

Because control experiments showed that LPMC cultured with IL-2 lysed P815 cells not labelled with antibody (mean cytotoxicity was 58.4% for eight specimens), cold target inhibition studies were performed to confirm that the lysis of P815-IgG targets by LPMC was due to ADCC rather than nonspecific cytotoxicity directed against nonimmunoglobulin receptors on P815 cells. Two LPMC specimens with IL-2-induced LAK activity against P815-IgG cells were studied with identical results (Fig. 6.3). Addition of cold P815 cells did not interfere with the lysis of P815-IgG targets, indicating that the lysis of P815-IgG and P815 target cells were mediated by different cytotoxic mechanisms. There was a similar lack of competition between P815-IgG and HL60 target cells which

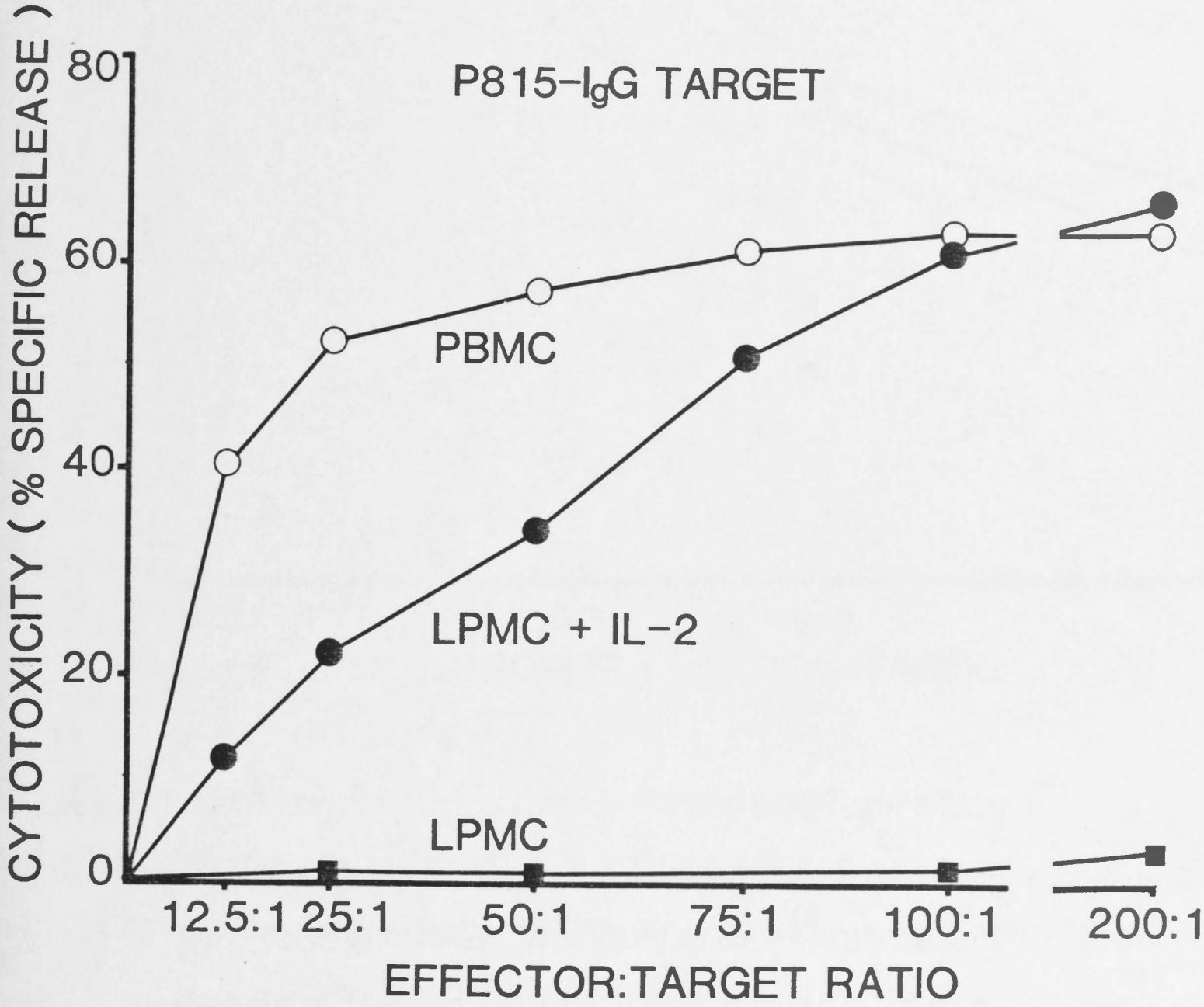

Figure 6.2 The ADCC of a representative LPMC population before (■) and after culture in the presence of IL-2 (●) compared with the spontaneous ADCC of PBMC from a normal donor (O). Cytotoxicity is expressed as % specific release from P815-IgG target cells at E:T ratios from 12.5:1 to 200:1.

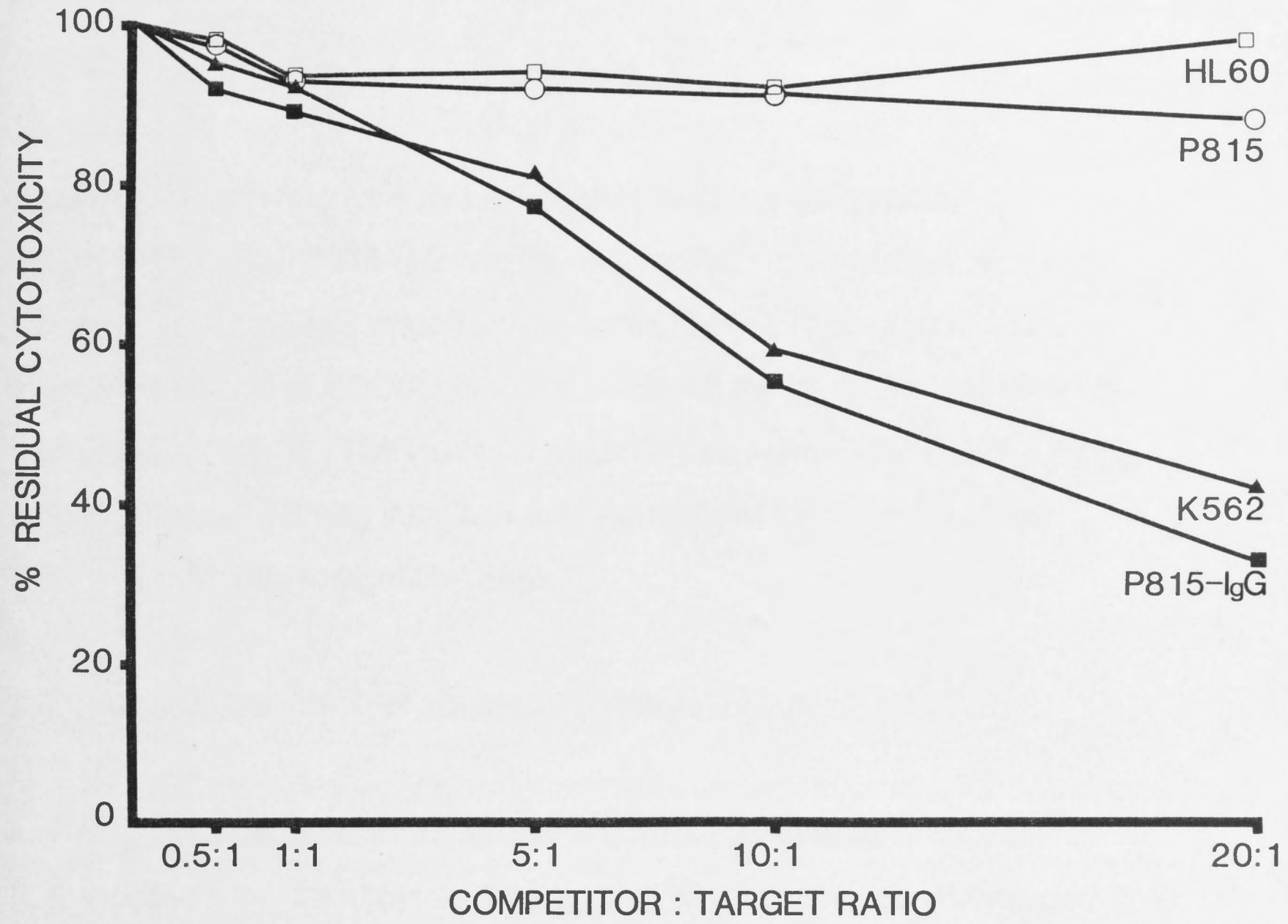

Figure 6.3 Cold target inhibition studies of the ADCC mediated by intestinal LAK cells. Cold P815, K562, HL60 or P815-IgG cells were added to microculture wells containing LAK cells and Cr^{51}-labelled P815-IgG cells. The degree of inhibition of ADCC was measured at competitor to target ratios from 0.5:1 to 20:1. The cytotoxicity of the LAK cell population against the target cell panel was as follows: P815-IgG (67.3%); P815 (49.8%); K562 (44.2%) and HL60 (55.4%). The addition of cold K562 or P815-IgG cells resulted in inhibition of lysis of P815-IgG targets by LAK cells, whereas added P815 and HL60 cells had no effect. The results shown are representative of experiments performed on two LPMC populations.

were also sensitive to lysis by LAK cells. Maximum inhibition of cytotoxicity against P815-IgG targets occurred after the addition of identical cold P815-IgG targets. With both LPMC specimens, comparable levels of inhibition of ADCC were also produced by the addition of cold K562 cells (Fig. 6.3). The lack of competition between P815-IgG and P815 cells in these cold target inhibition assays confirms that intestinal LAK cells lyse P815-IgG targets by ADCC.

6.4 Phenotypic analysis of intestinal LAK cells mediating ADCC.

The effector cells in eight LPMC populations mediating IL-2-induced ADCC and cytotoxicity against K562 cells were phenotyped by antibody and complement-mediated lysis (Table 6.1). Treatment with Leu-11b and complement resulted in variable reductions in ADCC which exceeded 30% of control levels in three of eight LPMC populations. Parallel studies of PBMC from five normal donors using Leu-11b showed greater than 90% loss of ADCC in all cases (two representative results are shown in Table 6.1). The ADCC of all four LPMC populations tested was reduced by more than 50% after incubation with OKT11 but was unaffected in all cases after using OKT3. Analysis of the same LPMC populations using K562 cells as targets showed consistent decreases in cytotoxicity only after treatment with the OKT11 antibody. After incubation with Leu-11b, two LPMC populations out of the eight tested showed minor losses of activity against K562 targets (both less than 25%). These

Table 6.1 The phenotype of intestinal LAK cells mediating ADCC.

Intestinal disorder	P815-IgG target				K562 target			
	C control[a]	OKT3	OKT11	Leu-11b	C control	OKT3	OKT11	Leu-11b
Carcinoma	37.8%[b]	34.1%	13.3%	31.1%	35.5%	31.9%	14.2%	30.0%
Carcinoma	23.9%	27.8%	4.2%	10.0%	16.4%	-[c]	0.0%	12.2%
Carcinoma	24.8%	27.4%	0.0%	26.4%	40.0%	43.1%	20.3%	41.4%
Diverticular disease	56.8%	66.4%	27.7%	37.2%	-	-	-	-
Carcinoma	44.6%	-	-	32.2%	25.5%	22.7%	12.8%	18.8%
Carcinoma	35.8%	-	-	37.3%	34.4%	-	-	35.5%
Carcinoma	75.8%	-	-	76.0%	72.8%	-	-	68.8%
Carcinoma	26.8%	-	-	18.4%	44.4%	-	-	34.9%
PBMC (PH)	33.2%	32.1%	18.9%	3.0%	-	-	-	-
PBMC (JS)	72.8%	75.8%	43.9%	3.9%	-	-	-	-

Intestinal LAK cells cytotoxic towards the target cells P815-IgG and K562 were phenotyped by antibody and complement-mediated lysis using the panel of monoclonal antibodies shown. Two representative results from similar studies of PBMC from five normal donors are shown for the P815-IgG target.

(a) Control values were from cells treated with complement alone before cytotoxicity testing.

(b) Cytotoxicity is expressed as % specific release from target cells at an E:T ratio of 50:1.

(c) Experiment not performed.

experiments demonstrate that IL-2-induced ADCC is mediated by LPMC which are predominantly $CD2^+3^-16^-$. In three out of eight specimens, $CD16^+$ cells mediated a significant but minor proportion of the ADCC. The $CD2^+3^-16^-$ LPMC cytotoxic towards K562 cells have been characterized previously as intestinal LAK cells (Chapter 3).

IV. Discussion

This investigation of ADCC in the human intestinal mucosa confirms the findings of previous studies showing that freshly isolated LPMC failed to mediate significant levels of ADCC (350,352,363,364). After exposure to IL-2 in culture however, LPMC acquired high levels of ADCC against P815-IgG targets comparable to those found in the peripheral blood of normal donors. This finding is similar to the IL-2-induced generation of intestinal LAK cells from cultures of LPMC (27,321,354) and therefore raises the possibility that LAK cells mediate the ADCC described in the present study. The majority of LPMC mediating IL-2-induced ADCC were $CD2^+3^-16^-$ which is the same phenotype reported in Chapter 3 for intestinal LAK cells cytotoxic towards K562 cells. This profile of surface markers distinguishes the LPMC effector cell from the $CD2^+3^-16^+$ NK cell and the $CD2^+3^+16^+$ Tγ cell which are regarded as the only lymphocyte types capable of mediating spontaneous ADCC in the peripheral blood (15,17). It is probable therefore, that the effector cell mediating IL-2-induced ADCC is the intestinal LAK cell. Whether both

forms of cytotoxicity are mediated by the same cell or by different subpopulations of LAK cells was not addressed in this study.

Although it is generally accepted that only lymphocytes with $CD16^+$ Fc receptors mediate ADCC, the findings of the present study and of others (292,437) suggest that activated killer cells which lack the CD16 determinant may also be capable of ADCC. An explanation for the ADCC of intestinal LAK cells may be that they express functional Fc receptors which are antigenically different from those on NK cells. This hypothesis is supported by the variation in antigen expression on the Fc receptors of lymphocytes, granulocytes and monocytes (436).

The lack of competition between P815 and P815-IgG cells in the cold target inhibition studies suggests that intestinal LAK cells lyse P815-IgG targets by ADCC. The reason for the inhibition of P815-IgG target lysis by K562 cells is unclear. The K562 cell expresses Fc receptors which may block the ADCC of P815-IgG targets by binding to the rabbit IgG fixed to the targets (438-440). However, there was no evidence of inhibition by the P815 and HL60 cell lines which express apparently similar Fc receptors (440,441). The blocking properties of the K562 cell lines may be explained by a higher Fc receptor density or affinity for rabbit IgG compared to P815 and HL60 cells. Alternatively, if the same LAK cell can mediate both LAK activity and ADCC, K562 competitors may be the most effective at blocking ADCC by steric hindrance. Complete explanation of the observations reported here will require investigation of the IgG binding properties of the K562, P815 and HL60 cell lines and the

use of competing target cells which do not express Fc receptors.

In three out of eight LPMC specimens treated with IL-2, minority populations of $CD16^+$ lymphocytes mediated a variable but significant proportion of the ADCC. This population may originate from cells belonging to the NK/K lineage which mediates ADCC through $CD16^+$ Fc receptors. Although immunohistochemical studies suggest that $CD16^+$ cells are infrequent in the intestine (321,356), small numbers of cells present in some specimens may proliferate in response to IL-2, resulting in an expanded population of $CD16^+$ LAK cells (78). The cytotoxic properties of these cells was not examined in detail, but previous studies suggest that $CD16^+$ NK cells exposed to IL-2 in culture acquire the unrestricted cytotoxic properties characteristic of LAK cells (309). Thus the $CD16^+$ cells described in this study may be NK cell-derived LAK cells which are known to be capable of mediating ADCC (292). Despite the presence of $CD16^+$ cells mediating ADCC against P815-IgG cells in three LPMC populations, phenotypic studies of the same specimens did not detect significant numbers of $CD16^+$ cells when tested against K562 target cells. This finding may be explained by differences in the susceptibility of target cells to lysis by cytotoxic cells and suggests that P815-IgG cells are more sensitive than K562 cells as targets for the detection of $CD16^+$ LAK cytotoxic cells. Previous studies of intestinal lymphocytes have also found that the results of cytotoxicity testing can be influenced by the nature of the cells used as targets (360). Contrary to the findings of most studies, Targan et al reported significant levels of spontaneous

cytotoxicity in the intestinal mucosa against intestinal epithelial cells but not NK cell-susceptible cell line targets. The implication of these results is that the characterization of cytotoxic cells in the intestinal mucosa may be affected by the choice of target cell.

The potential for LPMC to mediate ADCC in vivo would depend partly upon the availability of IL-2 within the microenviroment of the intestinal mucosa. In response to mitogen stimulation in vitro, LPMC from histologically normal or inflamed intestinal mucosa release biologically significant amounts of IL-2 (413,414,435). Therefore under conditions favouring the local production of IL-2, LPMC in the guise of LAK cells may protect against microbial invasion or neoplastic transformation of the normal intestinal mucosa. Through their ability to mediate ADCC, intestinal LAK cells may also have adverse effects in diseases such as IBD. High levels of circulating antibody against intestinal epithelial cells and other components of the bowel have been documented in patients with IBD (442). It is possible that by interacting with these antibodies, LAK activity may be directed against cellular constituents of the intestine, thus contributing to mucosal injury and ongoing inflammation in IBD.

The data presented in this study show that LPMC isolated from the intestinal mucosa mediate significant levels of ADCC only after IL-2-induced transformation into LAK cells. The cell population mediating ADCC consisted predominantly of $CD16^-$ LAK cells with a minor contribution by $CD16^+$ cells. The ability of intestinal LAK cells to mediate ADCC complements their nonspecific cytotoxic properties by

providing high levels of antibody-directed specific cytotoxicity against cellular or microbial targets.

CHAPTER 7.

Interleukin 2 production by lamina propria mononuclear cells in colon carcinoma and nonmalignant intestinal disorders.

7.1 Introduction

Interleukin 2 (IL-2) is a 15 kD molecular weight glycoprotein which plays a pivotal role in the regulation of key elements of the cellular immune system (443). In response to antigenic or mitogen stimulation, T cells from both the helper and cytotoxic/suppressor subgroups secrete IL-2 (444,445). The prime function of IL-2 is as a growth and differentiation factor for T cells (446-448) but it has been recognised that activated B cells also proliferate and differentiate in response to IL-2 (449-450). In addition to its effects on T and B cells, IL-2 is also the major stimulus for the proliferation of natural killer (NK) and lymphokine-activated killer (LAK) cells (27,451) which possess antineoplastic, antimicrobial and regulatory properties (27,197,452). Recent evidence also suggests that IL-2 may play a part in the activation of macrophages (453). Under the influence of IL-2, subpopulations of peripheral blood mononuclear cells (PBMC) secrete immunoregulatory lymphokines, including gamma interferon (255,454) and tumor necrosis factor and lymphotoxin (455).

Following characterization of IL-2 and its effects in the normal

immune system, there has been mounting speculation concerning the role of IL-2 in the pathogenesis of a wide range of human diseases. Defective production of IL-2 by PBMC has been reported in association with primary immunodeficiency states (456), autoimmune diseases (457,458) and malignancy (459). Recent studies have focused on diseases of unknown aetiology such as inflammatory bowel disease, where reduced production of IL-2 by intestinal lymphocytes is one of several documented immunoregulatory abnormalities (369,414,460).

Because PBMC populations are most accessible for investigation, relatively little is known about IL-2 production by lymphoid cells underlying mucosal surfaces. Preliminary studies report that lamina propria mononuclear cells (LPMC) isolated from the human intestinal mucosa are capable of producing significant amounts of IL-2 after mitogenic stimulus (413,414,435). However little is known about the effects of neoplastic or inflammatory disease on the production of IL-2 by intestinal mucosal lymphocytes. The mucosal production of IL-2 may be important in limiting the development of colorectal carcinoma as intestinal NK and LAK cells with antineoplastic properties are IL-2-dependent (27,71,451).

The purpose of the present study was to determine whether LPMC from the human intestine were capable of producing IL-2 in biologically significant amounts. Populations of LPMC from resected intestinal mucosa were stimulated with mitogens and the culture supernatants were assayed for IL-2 activity. A secondary objective was to explore the possible

relationship between intestinal IL-2 production and both the presence and extent of colorectal carcinoma. The levels of IL-2 production by LPMC were therefore compared in histologically normal specimens of intestinal mucosa resected for nonmalignant conditions or for colorectal carcinomas which were categorized according to their degree of spread.

II. Materials and Methods.

7.1 Patient details.

Samples of colonic mucosa were obtained from 26 patients at the time of surgery for colorectal carcinoma or for nonmalignant conditions excluding inflammatory bowel disease. The extent of tumour spread in the 18 subjects with carcinoma was staged according to the Duke's classification (461) as follows : Duke's A (three patients); Duke's B (five patients); Duke's C (eight patients) and Duke's D (two patients). The nonmalignant conditions resected included diverticular disease (three patients); angiodysplasia of the caecum (one patient); villous adenoma (one patient); polyposis coli (one patient); rectal prolapse (one patient) and ileocaecal lipoma (one patient). All samples of colonic mucosa were histologically normal and were obtained from areas at least five cm away from tumours or polyps.

7.2 Induction of cytokine production from mononuclear cells.

Mononuclear cells were stimulated to produce cytokines by incubation at a concentration of 5×10^6 cells/ml in RPMI 1640 medium (Flow laboratories, Virginia, USA) supplemented with 5 or 20 µg/ml of phytohaemagglutinin-P (PHA) (Commonwealth Serum Laboratories, Melbourne, Australia) without added serum. After incubation for 1 h at 37^oC in an atmosphere of 5% CO_2 in air, mononuclear cells were washed 3 times and cultured in medium alone at a concentration of 2×10^6 cells/ml in 24 well flat bottom culture plates (Linbro, Flow laboratories, Virginia, USA). Following incubation for 24 h, supernatants were aspirated from wells and stored at -20^oC until assayed. Five specimens treated by mitogen stimulation were also tested in parallel for spontaneous cytokine production by incubation in medium without PHA.

7.3 Assay for interleukin 2 activity.

The concentration of IL-2 in culture supernatants was measured in a proliferation assay using a cloned IL-2-dependent mouse cytotoxic T cell line (CTLL-2) (462). Serial dilutions of culture supernatant were combined with 10^4 CTLL-2 cells in triplicate in 96 well flat bottom microculture plates (Nunc Intermed, Denmark) in a final volume of 0.1 ml of medium supplemented with 10% foetal bovine serum (FBS) and 10^{-5}M 6-mercaptoethanol. After incubation for 18 h, the cultures were pulsed

with 1 μCi of tritiated thymidine for 6 h, harvested on glass fibre paper using a cell harvester (Skatron, Flow laboratories, UK) and measured for tritiated thymidine uptake in counts per minute (cpm) using a scintillation counter (Tri Carb, Packard, Illinois, USA). In each assay, control wells measured the background proliferation of CTLL-2 in medium alone and also the response of CTLL-2 to PHA and the preparation of human recombinant IL-2 used to generate LAK cells in Chapters 3,4,5 and 6. The amount of IL-2 in culture supernatants was expressed as end point units (U) which were defined as the reciprocal of the sample dilution producing a level of CTLL-2 proliferation equal to the mean plus three standard deviations of the background proliferation for each assay. Minimum inter-assay variation in the estimation of IL-2 activity was achieved in the following ways (i) CTLL-2 cells from a stock population frozen at -70^{0}C were expanded under identical culture conditions for each assay in medium containing an optimum concentration of MLA144 CM (408) and (ii) each assay included titrations of known concentrations of human recombinant IL-2.

Details of LPMC and PBMC isolation and statistical analysis are given in Chapter 3.

III. Results

7.1 The production of interleukin 2 by LPMC.

All 26 LPMC populations studied produced detectable levels of IL-2 after mitogenic stimulus with 5 µg/ml PHA (Fig 7.1). For analysis of the IL-2 levels, LPMC populations were divided into nonmalignant and carcinoma groups and then specimens with carcinoma were further subdivided according to the degree of tumour spread. Specimens with the tumour confined to the bowel wall or mesentery were staged as Duke's grades A or B while further spread to mesenteric lymph nodes or distant organs was signified by Duke's grades C or D respectively. The IL-2 activity in culture supernatants from nonmalignant LPMC (mean activity 149 U) was significantly higher than the IL-2 levels produced by all specimens with carcinoma (mean activity 49 U; $p < 0.05$). When the carcinoma group was subdivided according to the extent of tumour spread nonmalignant IL-2 production was significantly higher compared to the Duke's A and B tumours ($p < 0.05$) but not to the Duke's C and D tumours ($p > 0.05$). Despite the significant difference demonstrated between nonmalignant and carcinoma specimens, there was substantial overlap between the amounts of IL-2 produced by individual LPMC populations from either group (Fig 7.1). Eleven LPMC populations treated with 20 µg/ml PHA produced two to three times the amounts of IL-2 resulting from exposure of LPMC to 5 µg/ml. The IL-2 activity of the nonmalignant LPMC (mean

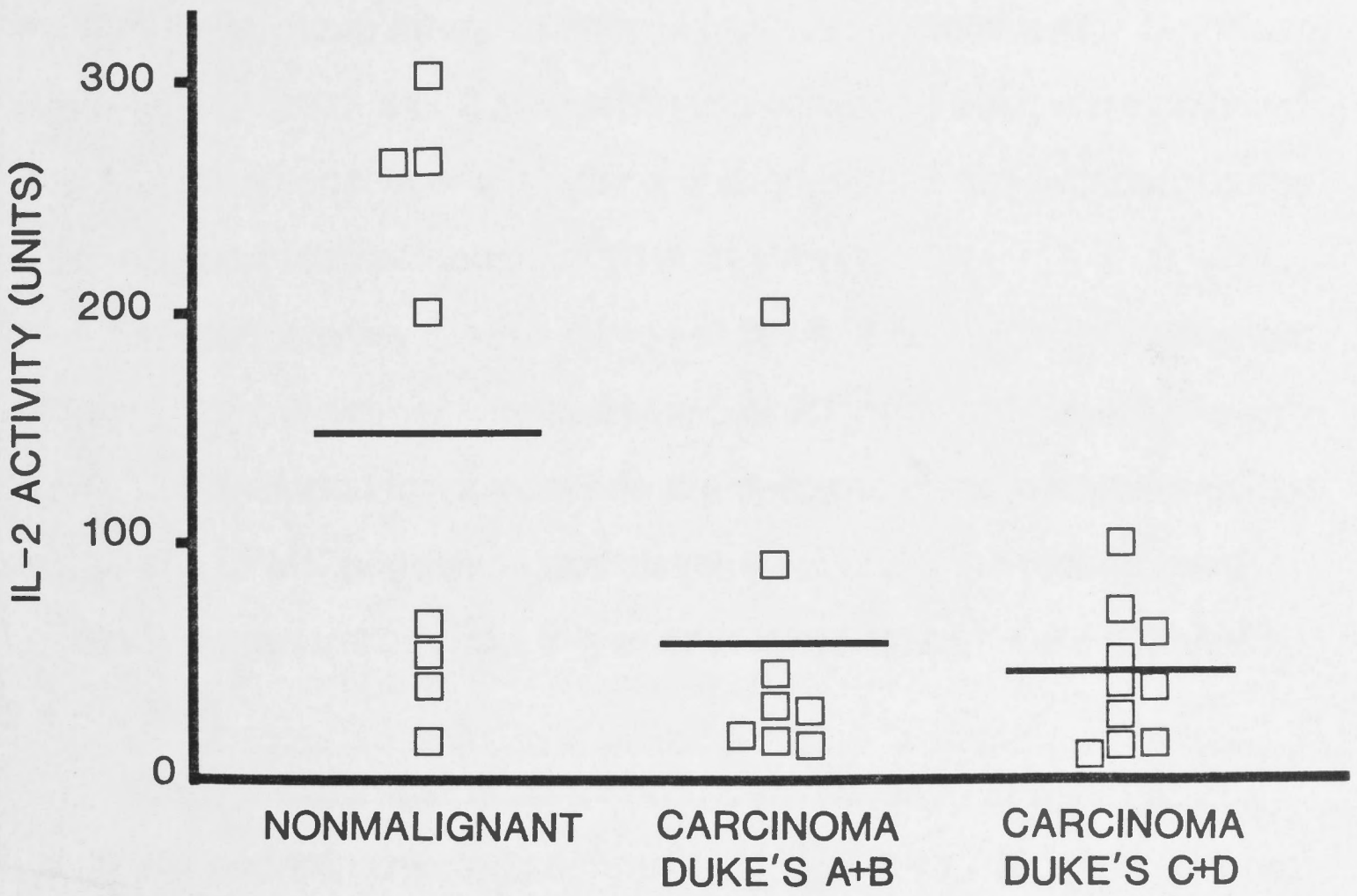

Figure 7.1 The IL-2 activity of culture supernatants from LPMC populations stimulated with 5 μg/ml PHA. The 26 populations are grouped into one nonmalignant and two carcinoma groups according to the extent of tumour spread as defined by the Duke's classification (see text). The activity of IL-2 is expressed as end point units (U) and the mean activity for each group is indicated by the horizontal bars. Significant differences were found between the nonmalignant group and all carcinoma specimens ($p < 0.05$) and the Duke's A and B subgroup ($p < 0.05$).

activity 326 U; four specimens) was again noted to be higher than that of the carcinoma group (mean activity 143 U; seven specimens). Significant levels of spontaneous IL-2 production (mean activity 30U) were detected in all five LPMC specimens (three nonmalignant and two with carcinoma) which were cultured without prior PHA treatment.

Representative titration curves of the IL-2 activity of nonmalignant and carcinoma specimens treated with 5 or 20 μg/ml of PHA are shown in Figure 7.2. Included for comparison are titrations of the spontaneous IL-2 activity of a LPMC population and also the activity of the recombinant human IL-2 preparation used to generate intestinal LAK cells (Chapters 3,4,5 and 6).

Control experiments performed as part of each assay of IL-2 activity showed that concentrations of PHA from 0.1 to 100 μg/ml did not affect the thymidine uptake of CTLL-2 (data not shown). Finally, to exclude an effect of the enzymatic isolation procedure on the production of IL-2 by LPMC, PBMC from two normal donors were exposed to the same treatment before induction of IL-2 production. In both cases there was no significant difference between the levels of IL-2 produced by PHA-stimulated PBMC either before or after enzyme treatment (data not shown).

These results indicate that LPMC produce significant levels of IL-2 spontaneously in small amounts and in greater quantities after stimulation with the mitogen PHA. Although there was considerable overlap between the levels of IL-2 from different groups, LPMC isolated

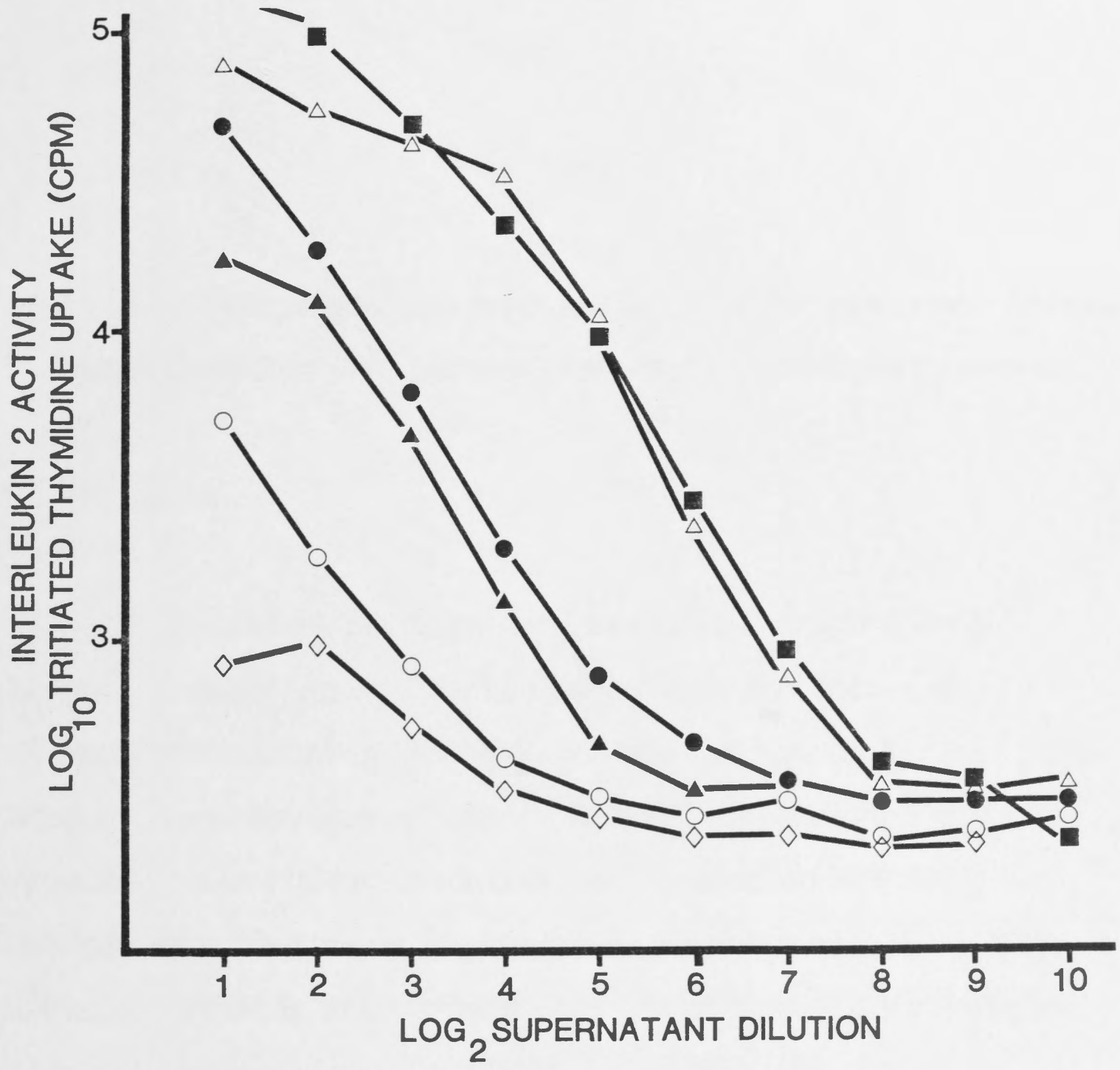

Figure 7.2 Titration curves of IL-2 activity in culture supernatants of LPMC stimulated with 5 or 20 µg/ml PHA. The proliferation assay measured the uptake of tritiated thymidine in cpm (vertical axis) by the CTTL-2 cell line. Serial dilutions (Log_2) of the sample are shown on the horizontal axis. The titrations shown are representative results obtained with LPMC from a carcinoma specimen (O 5µg/ml PHA ; ● 20 µg/ml PHA) and a nonmalignant specimen (diverticular disease) (▲ 5µg/ml PHA ; Δ 20 µg/ml PHA). Also shown are results from the assay of a human recombinant IL-2 preparation (■) and the culture supernatant of a LPMC population (from the specimen with colon carcinoma shown) incubated in medium without prior PHA stimulation (◇).

from nonmalignant specimens produced significantly higher mean levels of IL-2 when compared with specimens resected for colorectal carcinoma.

IV. Discussion.

Analysis of the phenotypic and functional characteristics of intestinal mucosal lymphocytes has proven to be an effective approach towards the investigation of immunoregulation at mucosal surfaces (400). Although numerous studies have shown that intestinal lymphocytes influence cellular proliferation and antibody production (460,463), very little is known about their ability to produce lymphokines (413,414,435). In the present study, another facet of the immunoregulatory potential of intestinal lymphocytes was investigated by testing their capacity to produce IL-2. The important initial observation of this study was that LPMC produce significant levels of IL-2, both spontaneously and after mitogen stimulation. The spontaneous production of IL-2 is consistent with the activated state of LPMC (385,389,414) and contrasts with PBMC which produce IL-2 only after stimulation with antigens or mitogens (445). The LPMC population producing IL-2 is therefore potentially capable of influencing the differentiation and function of IL-2-responsive T cells, B cells and nonspecific cytotoxic cells in the intestinal mucosa. The LPMC population responsible for IL-2 production was not characterised in this or previous studies, but investigations using PBMC suggest that T cells of either helper or suppressor phenotype (444,445) or cells related to the NK

lineage (464) may be responsible.

A further aim of this study was to investigate the role of mucosal IL-2 production in the pathogenesis of colorectal carcinoma. The presence of significant levels of IL-2 in the intestinal mucosa may protect against malignancy, as colon carcinoma cells are lysed in vitro by LAK cells (197) and possibly cytotoxic T cells (206), both of which are IL-2-dependent. The present finding of reduced IL-2 production in relation to colon carcinoma has been reported previously (413), but other studies have failed to confirm such an association (414,435). This disparity in findings may be partly attributable to differences in the cellular content of LPMC populations and the type of mitogen used to stimulate IL-2 production (414). The significantly lower levels of IL-2 production found in specimens resected for carcinoma could be due to reduced IL-2 production on a per cell basis and/or to smaller numbers of IL-2 producing cells. This finding may indicate the existence of a primary defect in mucosal IL-2 production which could predispose towards the development of malignancy. Alternatively, the lower levels of mucosal IL-2 observed in colorectal carcinoma may be an immunosuppressive effect secondary to the presence of malignancy. A range of tumours including colorectal carcinoma are reported to release poorly characterized high molecular weight factors which inhibit lymphocyte proliferation (465-467) and IL-2 production (466,468). In addition, colorectal carcinomas contain detectable amounts of prostaglandin E which also impairs IL-2 production by PBMC (469,470). As a consequence of this acquired defect in IL-2 production, localised and

potentially curable colorectal carcinomas may undergo accelerated progression to more extensive or disseminated tumours.

The production of IL-2 locally in the intestinal mucosa may be of importance in the pathogenesis of inflammatory bowel disease (IBD), where abnormalities in the immunoregulatory properties of both peripheral blood and intestinal lymphocytes are prominent findings (431,460). However, the existence of a defect in IL-2 production by LPMC in IBD is so far unproven, as insufficient numbers of IBD specimens have been examined and compared with histologically normal mucosal specimens from intestinal disorders other than IBD. Independent studies of IBD have reported normal levels of IL-2 production by PHA-stimulated LPMC (414,435), but when phorbyl-myristic acetate was used as the stimulus, IL-2 production was found to be depressed (414). The question of IL-2 availability in the IBD mucosa requires further investigation, as regulatory T cell dysfunction secondary to reduced levels of this lymphokine may perpetuate the chronic inflammation typical of this disorder (414).

Interleukin 2 was the only known T cell growth factor until the recent description of interleukin 4 (IL-4), which is also known as B cell stimulatory factor-1 (471). Although its major effect is on B cells, this 20 kD molecule is capable of stimulating T cell proliferation independently of IL-2 through interaction with a specific receptor. The effect of IL-4 on the system used to assay IL-2 in this chapter is unknown, but CTLL lines are reported to be responsive to IL-4, although to a lesser

extent than IL-2 (471). At the time the experiments reported here were performed, pure preparations of IL-4 were unavailable. Future studies of IL-2 production by LPMC will have to assess the effect of IL-4 on the IL-2 assay and attempt to detect the presence of IL-4 in LPMC supernatants by the use of ion exchange chromatography and specific inhibitory antisera.

The induction of IL-2 production by mitogenic stimulation in vitro may not reflect the in vivo production of IL-2 by LPMC in the intestinal mucosa. A critical factor influencing the amount of IL-2 induced in culture is the proportion of macrophages present. In cultures of PBMC, excess numbers of monocytes inhibit IL-2 production by the prostaglandin E_2-induced activation of suppressor T lymphocytes (472), but on the other hand, optimum IL-2 production requires a minimum presence of monocytes (1-5%) to provide a source of interleukin 1 for T cell activation (472). The possible effect of intestinal macrophages on IL-2 production by LPMC has not been systematically studied, although Fiocchi et al (414) found that the removal of macrophages from LPMC populations made no difference to IL-2 production. In the present study, the relatively constant proportion of intestinal macrophages (17%) present in the LPMC populations (Chapter 3) may have influenced the total amounts of IL-2 produced, but not the differences observed between nonmalignant and carcinoma specimens. Although control experiments showed no effect of the isolation procedure on IL-2 production by PBMC, the large amounts of prostaglandin liberated during isolation of LPMC (358) may also affect their ability to produce optimum levels of IL-2. Other variables affecting IL-2 production include

the amount and type of mitogenic stimulus and the concentrations of serum used in culture (473). The amount of IL-2 measured in culture supernatants is also determined by the balance between lymphokine production and consumption by activated lymphocytes (474). This observation is pertinent to the study of LPMC populations which contain activated T cells (389,414). All these variables must be considered in the interpretation and comparison of IL-2 production by mononuclear cell populations.

The findings of the present study confirm that LPMC produce biologically significant levels of IL-2 and suggest that cells with this property have a potentially important immunoregulatory function in the intestinal lamina propria. A deficit of mucosal IL-2 production associated with colon carcinoma may have important implications because of deleterious effects on local cell-mediated immune mechanisms within the intestinal mucosa. Further investigation of IL-2 production by LPMC may yield important insights into the pathogenesis of colorectal carcinoma and IBD.

CHAPTER 8.

Partial characterization of the colony stimulating factor activity produced by lamina propria mononuclear cells.

I. Introduction

The colony stimulating factors (CSF's) are a family of glycoproteins which were first recognized as essential growth factors for granulocyte and macrophage colonies derived from bone marrow precursor cells cultured in semi-solid agar (475). In the human, purification and molecular cloning techniques (476-479) have so far lead to the identification of 4 separate molecules with CSF activity, specific either for granulocytes (G-CSF), macrophages (M-CSF) or for both cell lineages (GM-CSF and IL-3). As well as supporting the growth and differentiation of granulocytes and macrophages, the CSF's are also responsible for modulating numerous functions of the mature cells (480,481). Antibody-dependent killing of tumour cells and helminth parasites by neutrophils and eosinophils is markedly enhanced by the CSF's (482-484). Increased phagocytosis of yeast particles, augmented superoxide production and the upregulation of type 3 complement receptors (CR3) on granulocytes are also attributable to the CSF's (484-486). Many cellular and humoral components of the inflammatory response may be modified by CSF's which are produced in high concentrations at the site of

experimentally-induced inflammation (487). The chemotaxis of neutrophils and their immobilization and survival at inflammatory foci are CSF-dependent (484,485). Moreover, CSF's are able to stimulate the secretion of potent inflammatory mediators including leukotriene C_4 (483), tumour necrosis factor (488), interferon (489), interleukin 1 (490) and plasminogen activator (491).

Although CSF production has been detected in a range of organs in the mouse (492), little is known about the endogenous production of CSF's within the human intestinal mucosa. By activating the cytocidal properties of granulocytes and macrophages, mucosal CSF production may be important in maintaining defence mechanisms against infection or the development of neoplasia. Furthermore, CSF's may be an important regulator of disease activity in IBD, which is characterized by mucosal infiltrates of granulocytes and macrophages (493). Because of the potential importance of CSF production in the normal or diseased bowel, the present study tested the ability of LPMC to produce CSF activity. Histologically normal specimens of intestinal mucosa resected either for colorectal carcinoma or benign disorders were tested for CSF production by LPMC and the levels were correlated with the presence or absence of malignancy. The type of CSF produced was also investigated by using hydrophobic interaction chromatography which is able to differentiate between the activities of GM-CSF and G-CSF (494). The results of this investigation demonstrate that LPMC from the human intestinal mucosa produce significant levels of CSF activity which are not affected by the

presence of an underlying malignancy.

II. Materials and Methods.

The details of the patients studied and methods of mononuclear cell isolation, induction of lymphokine production and statistical analysis are outlined in Chapter 7.

8.1 Assay of colony stimulating factor activity.

Supernatants from cultures of mitogen-stimulated LPMC were tested for colony stimulating factor (CSF) activity in a proliferation assay using human bone marrow-derived stem cells (Hapel et al, unpublished data). Previous experiments using recombinant preparations of G-CSF and GM-CSF have shown that the bone marrow cells used in the assay are reponsive to at least both of these factors. Human bone marrow cells were obtained from operative specimens of medullary bone removed from patients undergoing joint replacement or corrective procedures for scoliosis. The marrow space was repeatedly flushed and aspirated with phosphate buffered saline (PBS) and the resulting cell suspension centrifuged over Ficoll-Hypaque gradients (Pharmacia, Uppsala, Sweden) at 500g for 30 min at room temperature. Interface cells were harvested, washed three times and then cultured in flat bottom, six well culture plates (Costar, Mass, USA) at a concentration of 5×10^6 cells/ml in

Iscove's minimal essential medium (IMEM) (Flow Laboratories, Virginia, USA) supplemented with 20% foetal bovine serum (FBS) and 10^{-5}M hydrocortisone (Sigma Chemical Co., St. Louis, MO). After two to three weeks' culture (with changes of medium every 3 days), the semiadherent cells were removed by repeated gentle flushing and aspiration and fractionated by discontinous gradient centrifugation over three densities of Percoll (Pharmacia, Uppsala, Sweden) as follows : 30% (3ml); 40% (3ml) and 50% (3 ml). After centrifugation at 500g at room temperature for 30 min, the cell interface above the 50% Percoll was harvested, washed and resuspended in IMEM medium supplemented with 20% FBS and 10^{-5}M hydrocortisone.

The assay for CSF activity was performed in 96 well, flat bottom microculture plates (Nunc Intermed, Denmark) by combining IO^4 bone marrow cells from the 50% Percoll fraction in supplemented IMEM medium with serial dilutions of LPMC culture supernatants in a final volume of 0.1 ml. After culture for 48 h at 37^{o}C in an atmosphere of 5% CO_2 in air, the cells were pulsed with 1 μCi of tritiated thymidine for 18 h, harvested and counted as described in Chapter 7. Background proliferation of bone marrow cells was determined in the presence of medium alone. Control wells in each assay contained serial dilutions of conditioned medium from the GCT and 5637 cell lines which are known sources of GM-CSF and G-CSF (495,496). In accompanying experiments, bone marrow stem cells were shown to be unresponsive to concentrations of PHA from 0.1 to 100 μg/ml (data not shown). The CSF activity of culture supernatants was expressed

as end point units (U) as described in Chapter 7. Reproducibility of results between assays using different preparations of bone marrow cells was ensured by the inclusion of standard preparations of conditioned medium from the GCT and 5637 cell lines in each assay.

8.2 Ion exchange chromatography.

Culture supernatants from two specimens (one carcinoma, one diverticular disease) were fractionated by ion exchange chromatography using diethylaminoethyl (DEAE)-sephacel (Pharmacia ,Uppsala, Sweden). Ten ml of DEAE-Sephacel was equilibrated in a 1.5 cm diameter column with starting buffer (0.05M Tris-HCl, pH 7.8) at a flow rate of 10 ml/h at room temperature. A 5 ml sample of culture supernatant was dialysed overnight at room temperature in 500 ml of starting buffer and was then applied to the column at a rate of 10 ml/h. The sample was followed by 30 ml of starting buffer and then 60 ml of an increasing linear salt gradient prepared from equal volumes of starting and finishing buffer (0.3M NaCl in 0.05M Tris-HCl, pH 7.8) and finally 40 ml of finishing buffer. The column was extensively re-equilibrated with starting buffer before the second lymphokine preparation was fractionated. The eluate was collected in 5 ml fractions and measured for conductivity, absorbance at 280 nm and CSF activity after passage through 0.45 μm filters (Millipore Corp., Bedford, Mass).

8.3 Hydrophobic interaction chromatography.

Fractions with high CSF activity obtained by ion exchange chromatography were pooled and concentrated in dialysis tubing in an air stream and were then dialysed overnight at room temperature against 100 volumes of starting buffer consisting of 1M ammonium sulphate; 0.1M sodium phosphate; 0.02% Tween 20 (Sigma Chemical Co., St Louis, Mo) and 0.02% sodium azide in distilled water. Eight ml of phenylsepharose (Pharmacia, Uppsala, Sweden) was equilibrated in a 1.5 cm diameter column with starting buffer at a flow rate of 10 ml/h at room temperature. The dialysed culture supernatant was applied to the column at 10 ml/h followed by 20 ml of starting buffer and a 60 ml decreasing linear gradient prepared from equal volumes of the starting buffer and the second buffer (0.02% Tween 20 and 0.02% sodium azide in distilled water). This was followed in turn by 20 ml of the second buffer; 60 ml of an increasing linear gradient prepared from equal volumes of second buffer and finishing buffer (70% ethylene glycol with 0.02% Tween 20 and 0.02% sodium azide in distilled water) and finally 40 ml of finishing buffer. Five ml fractions were measured for conductivity and absorbance and then dialysed against two changes of PBS before filtration and assay for CSF activity.

III. Results

8.1 The production of colony stimulating factor activity by LPMC.

Lamina propria mononuclear cells (LPMC) from all mucosal specimens tested produced detectable levels of CSF activity after stimulation with PHA. Twenty-one LPMC specimens treated with 5µg/ml PHA produced a mean CSF activity of 347 U (Fig 8.1). There was no significant difference between the levels of CSF produced by LPMC populations from specimens resected for nonmalignant conditions or for carcinoma (mean activities were 476 and 268 U respectively). Further subdivision of carcinoma specimens according to Duke's classification (461) before statistical analysis did not alter this result. Little overall increase in CSF production was achieved by treating LPMC with 20µg/ml PHA, which resulted in a mean CSF activity of 337 U for six specimens (four malignant, two benign). Comparatively large amounts of CSF were produced spontaneously by five LPMC populations which were cultured in medium alone (mean activity 109 U for two malignant and three benign specimens).

The titration curves of the CSF activity produced by representative LPMC populations from a nonmalignant and a carcinoma specimen are illustrated in Figure 8.2. In similar experiments to those outlined for IL-2 production by LPMC, the enzymatic isolation procedure had no effect on the PHA-stimulated production of CSF by PBMC populations from two normal

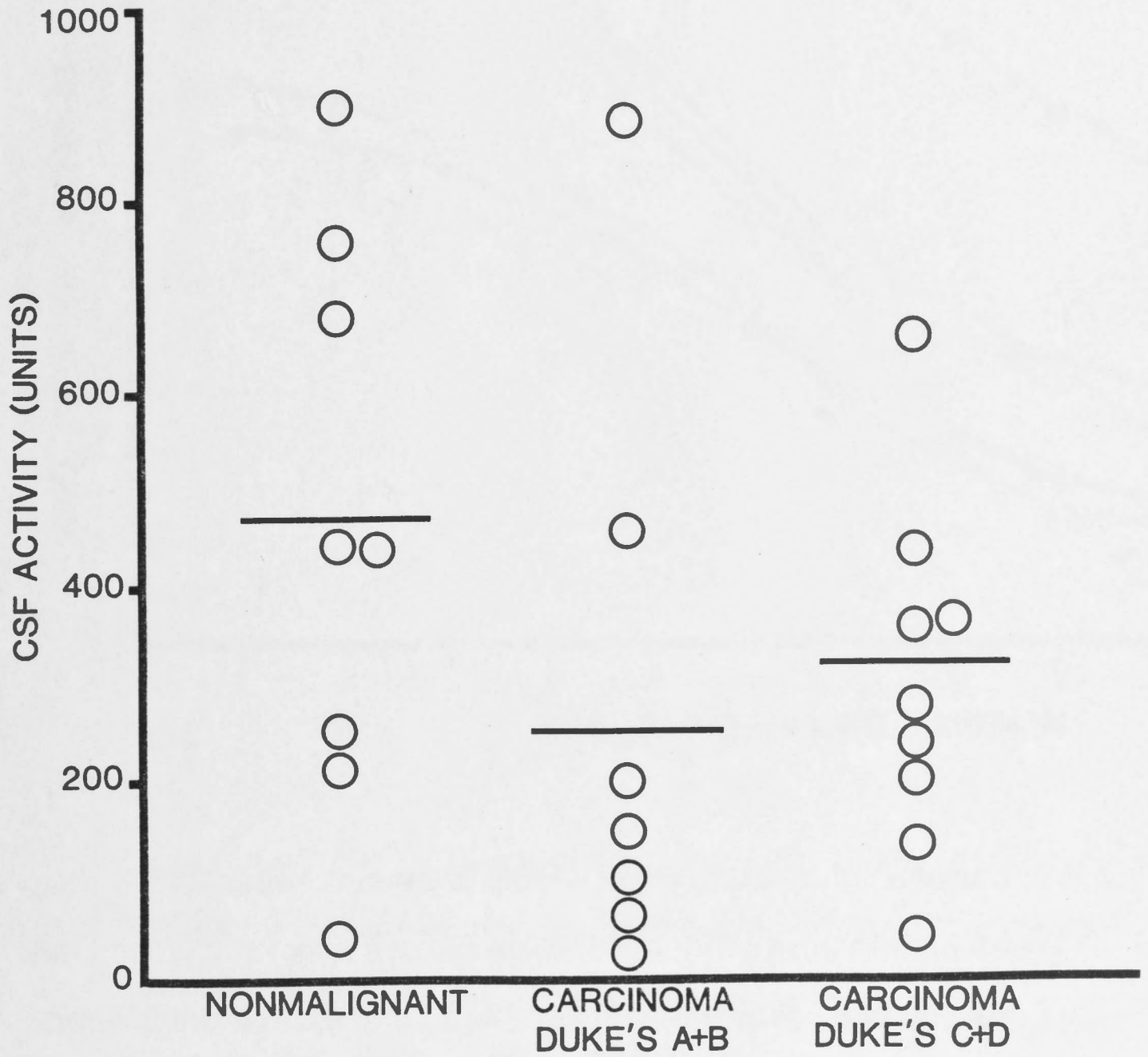

Figure 8.1 The CSF activities of culture supernatants from LPMC stimulated with 5 μg/ml PHA. The indications for colon resection are shown for each group of specimens. The activity of CSF is expressed as end point units (U) and the mean activities of each group are indicated by the horizontal bars. There was no significant difference between CSF titres for any of the groups shown.

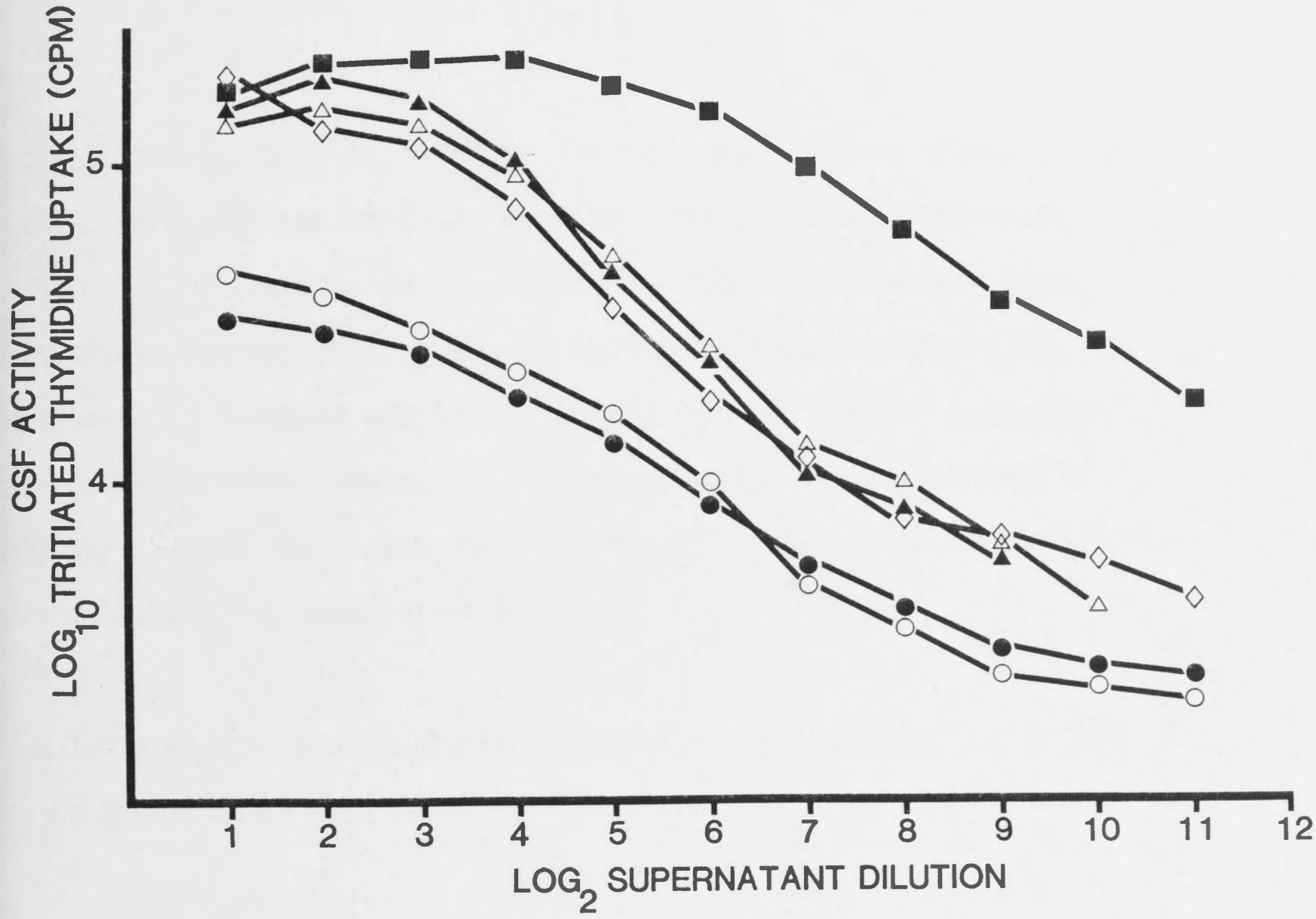

Figure 8.2 Titration curves of CSF activity in the culture supernatant of LPMC stimulated with 5 or 20 µg/ml PHA. The proliferation assay measured the uptake of tritiated thymidine in cpm (vertical axis) by cultured bone marrow cells. Sample dilutions (Log_2) are shown on the horizontal axis. The titrations shown are representative results obtained with LPMC from a carcinoma specimen (● 5µg/ml PHA ; O 20 µg/ml PHA) and a nonmalignant specimen (diverticular disease) (Δ 5µg/ml PHA ; ▲ 20 µg/ml PHA). Also shown are results from the assay of conditioned medium from the 5637 cell line (■) and the culture supernatant of a LPMC population (from the specimen with colon carcinoma shown) incubated in medium alone without prior PHA stimulation (◇).

control subjects (data not shown). The implication of these results is that LPMC are capable of producing other lymphokines in addition to IL-2. Although CSF was produced in greater amounts after PHA stimulation, LPMC also demonstrated significant spontaneous production of CSF. In addition, no correlation was found between the levels of CSF production and the presence or absence of malignancy. Experiments designed to identify the molecule(s) responsible for the CSF activity produced by LPMC are outlined in the following section.

8.2 Ion exchange and hydrophobic interaction chromatography of culture supernatants from PHA-stimulated LPMC.

Culture supernatants of PHA-stimulated LPMC were subjected to ion exchange chromatography by passage over a DEAE-Sephacel column in order to isolate the fractions containing CSF activity for further analysis. A single peak of CSF activity was detected in the eluate of culture supernatants from both nonmalignant and colon carcinoma specimens (Fig 8.3). In both specimens, the CSF activity bound to the column and was eluted in the linear salt gradient.

Hydrophobic interaction chromatography was then used to determine the nature of the CSF activity isolated by ion exchange chromatography. Previous studies have shown that the CSF activity in human placental conditioned medium could be separated by hydrophobic interaction chromatography into separate peaks of CSF activity originally

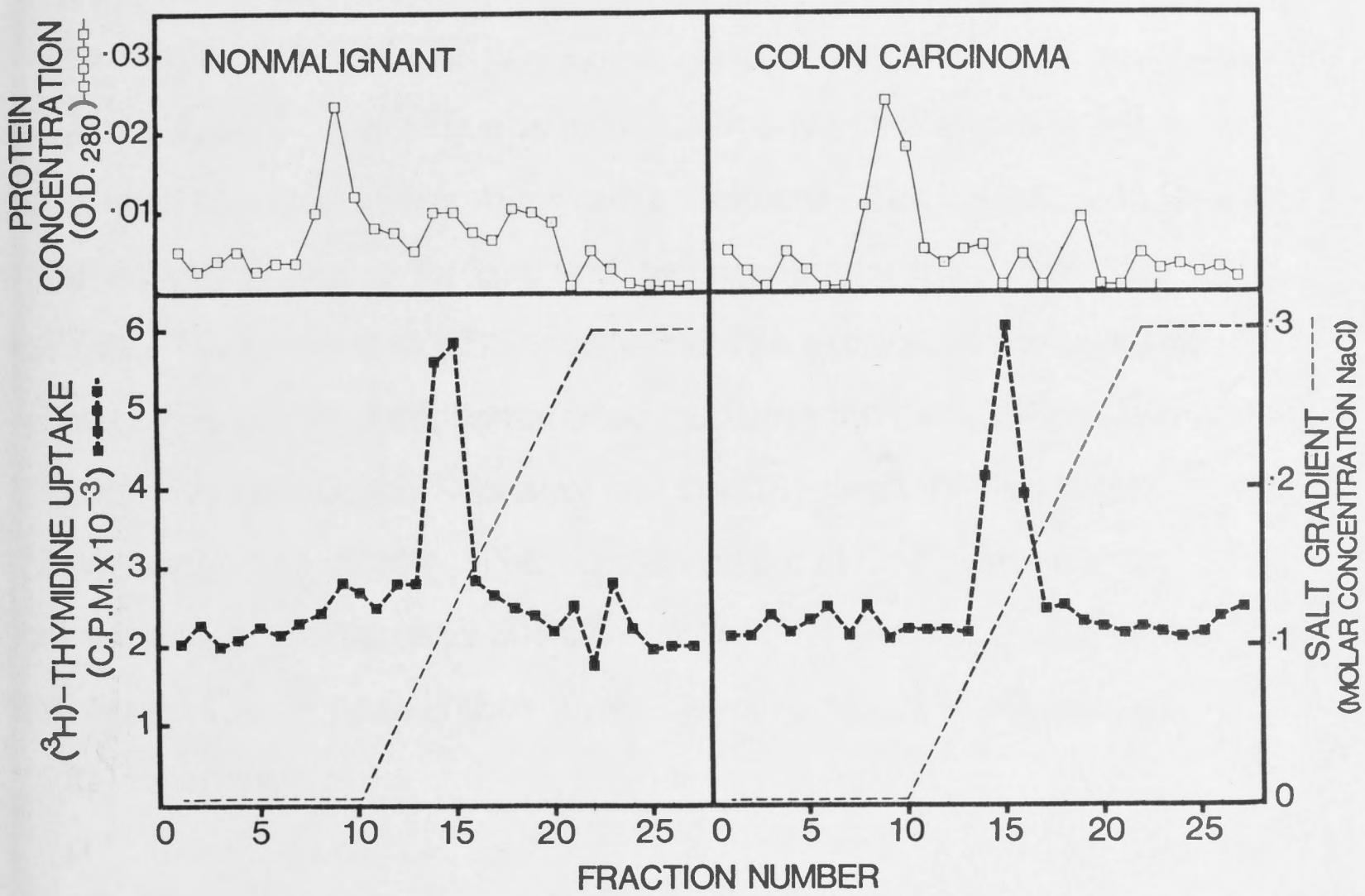

Figure 8.3 Ion exchange chromatography of culture supernatants from PHA-treated LPMC populations from a carcinoma and a nonmalignant specimen fractionated on a column of DEAE-Sephacel. A peak of CSF activity was detected in fractions eluted during application of the increasing linear salt gradient. CSF activity is expressed as counts per minute (cpm) of tritiated thymidine uptake by human bone marrow cells in the proliferation assay. A count of greater than 3,200 cpm indicates significant activity.

designated as CSF- α and CSF-ß (494). Subsequently, CSF- α and CSF-ß were identified as GM-CSF and G-CSF respectively (495,496). As shown in Figure 8.4, no CSF activity was detected in any of the eluted fractions for either the nonmalignant or malignant specimens. The low amounts of total CSF activity applied to the columns may account for the absence of activity. To confirm that CSF activity could be separated into separate peaks using this method, conditioned medium from the 5637 cell line was fractionated over DEAE-Sephacel and the CSF peak applied to the phenylsepharose column. Two discrete peaks of CSF activity which correspond to the unbound GM-CSF peak in the decreasing salt gradient and the bound G-CSF peak in the ethylene glycol gradient (494) were eluted (data not shown).

IV. Discussion.

The data presented in this study represent the first reported demonstration that factors with CSF activity are produced by mononuclear cells from the human intestinal mucosa. The identity of the CSF activity was unclear as hydrophobic chromatography of supernatants from cultured LPMC failed to detect the peaks characteristic of either GM-CSF or G-CSF. Other factors possibly accounting for this intestinal CSF activity include the recently cloned human IL-3 which combines the properties of both GM-CSF and G-CSF (479), or a novel undescribed CSF. The presence of factors such as IL-4, which can potentiate the effects of the CSF's on

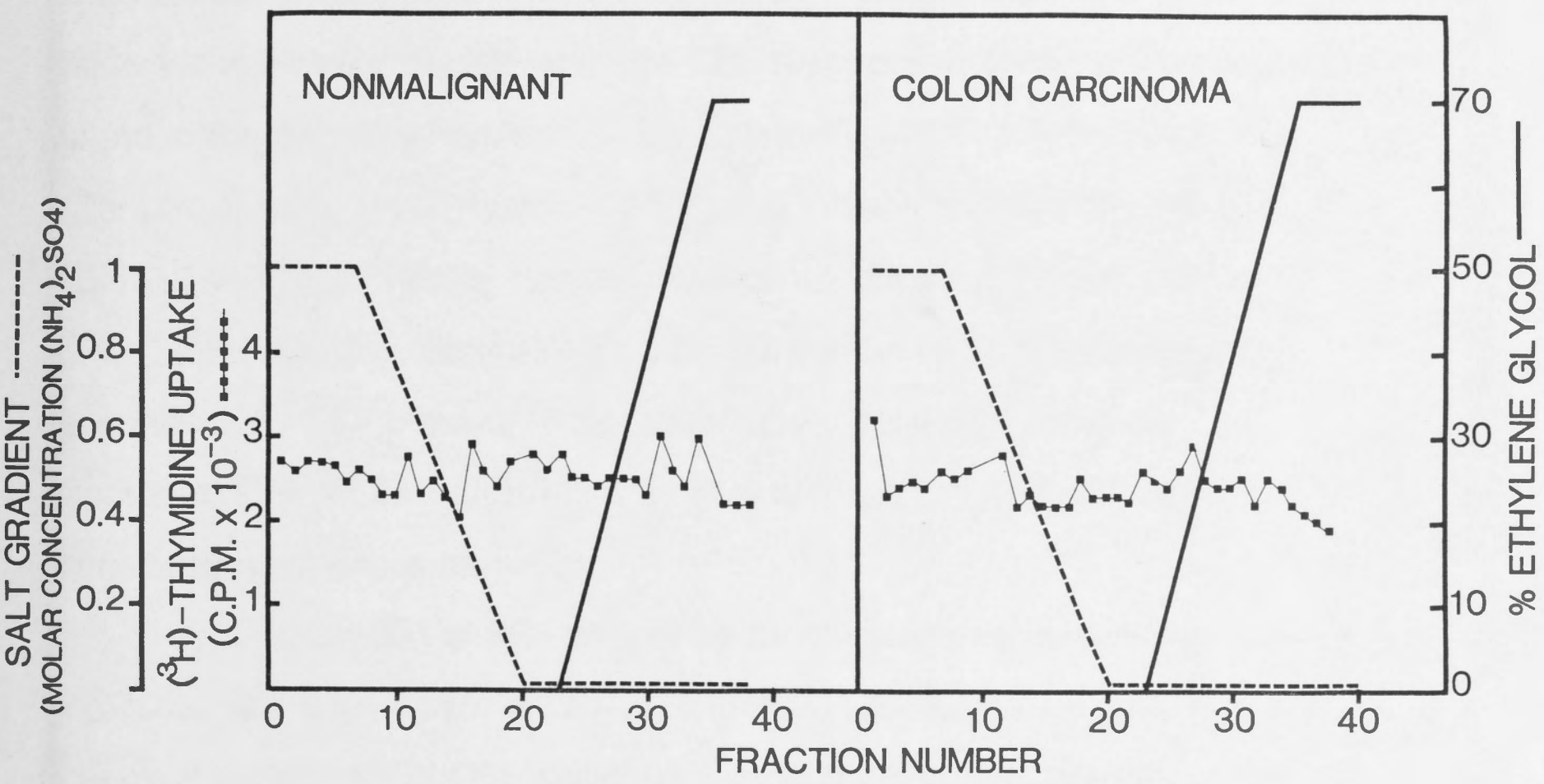

Figure 8.4 Hydrophobic interaction chromatography of CSF-containing fractions of LPMC supernatants from a carcinoma and a nonmalignant specimen on a phenylsepharose column. No detectable peaks of CSF activity were eluted during the decreasing linear salt gradient or the increasing ethylene glycol gradient. CSF activity is expressed as cpm of tritiated thymidine uptake by bone marrow cells. A value of greater than 2,800 cpm indicates significant CSF activity.

haemopoietic precursor cells is also possible (497). The identity of the cells responsible for intestinal CSF activity remains speculative, but likely producer cells include macrophages or T lymphocytes which are known to synthesize G-CSF and GM-CSF respectively (481). Other cells found in the intestinal mucosa but not isolated in LPMC preparations (Chapter 3) which may produce CSF in vivo include endothelial cells and fibroblasts (481). The augmentation of spontaneous CSF production by LPMC after mitogen stimulation is consistent with reports that low levels of constitutive CSF production by mononuclear cells can be rapidly increased after acute infection (481), treatment with endotoxin (492) or specific antigen exposure (498).

The production of CSF activity by mononuclear cells from the intestinal mucosa may serve several important functions. It is known that the significant levels of CSF activity detected in the serum originates from local organ producton (491,499). Intestinal CSF production may contribute therefore, to a circulating pool of factor which supports the growth, differentiation and function of any cell in the body expressing CSF receptors (480,481). Homeostatic mechanisms which regulate the intestinal response to microbes or antigenic stimulation may be dependent on local CSF production. Antimicrobial properties of granulocytes and macrophages enhanced by CSF's include chemotaxis, phagocytosis, superoxide production, complement receptor expression and antibody-dependent cellular cytotoxicity against helminths (482-486). The influence of CSF's on the phagocytic and receptor-mediated functions

of macrophages suggest that these factors may be important in antigen processing and presentation. Little is known about antigen presentation in the intestinal mucosa but CSF-responsive cells such as the intestinal macrophage may be involved.

The production of CSF activity by LPMC may protect against the development of neoplasia in the intestinal mucosa. Macrophages, eosinophils and polymorphonuclear leucocytes frequently infiltrate colorectal carcinomas, but is not known if these cell types can lyse colon carcinoma cells (500,501). Although CSF's augment the ADCC of tumour cells by granulocytes and macrophages, there is no evidence to suggest that this mechanism is operative in defence against colon carcinoma. In the present study, the lack of association between the levels of CSF production and the presence of carcinoma argues against the importance of CSF-supported effector cell mechanisms against colon carcinoma. Similar conclusions have recently been reported by Pullman et al (435).

The potent proinflammatory properties of CSF's may be important in the pathogenesis of inflammatory bowel disease (IBD). The ability of CSF's to first attract and then immobilize (484,485) neutrophils at sites of inflammation may contribute to the accumulation of granulocytes seen in the intestinal mucosa in active IBD. Once neutrophils are attracted to the intestinal mucosa, local CSF production may then lead to their activation and prolongation of life span (482-485). A recent study has documented significant CSF production by LPMC in IBD, but the levels of mitogen-stimulated CSF activity on a per cell basis were no different

from those produced by non IBD specimens (435). However, because of the increase in mucosal lymphoid cells in IBD, the overall amount of CSF activity in the intestinal mucosa may be increased (395). Thus, the augmented chemotaxis and phagocytosis of peripheral blood monocytes from patients with active IBD (389) may result from an enhanced intestinal production of CSF's and an increased release of CSF's into the circulation secondary to the effects of inflammation on the intestinal vasculature.

In summary, this study has demonstrated that LPMC from histologically normal intestinal mucosa produce one or more factors with CSF activity. The levels of CSF produced were not influenced by the presence of colorectal carcinoma, suggesting that variations in local CSF production by LPMC may not predispose towards the development of malignancy. The intestinal production of CSF activity deserves further investigation as it may have an important role in homeostasis of the normal intestine as well as in the pathogenesis of IBD.

CHAPTER 9.

Summation.

9.1 Aims and experimental approach.

The main aim of this thesis was to investigate the occurrence of nonspecific cytotoxic cells and cytokine production within the intestinal mucosa and then to relate possible abnormalities of these systems to the pathogenesis of colon carcinoma and inflammatory bowel disease (IBD). Intestinal natural killer (NK) and lymphokine-activated killer (LAK) cells were the focus of investigation because these cells have antineoplastic, antimicrobial and immunoregulatory functions which may be advantageous to the intestinal mucosa.

As malignant transformation of the human colonic epithelium is a common event, the possible role of nonspecific cytotoxic cells in eradicating early foci of tumour cells is an important issue. Furthermore, the strong implication from a large number of experimental and clinical studies is that NK and LAK cells are most effective against localised tumours in their early stages. Although these cells have a measurable influence on large tumour burdens in vivo, this effect is unlikely to be of major significance. The place of NK cells among the host's early defences against microbial pathogens is now well established. The ability of NK and LAK cells to destroy parasites, bacteria and viruses in both intracellular

and extracellular locations would be an obvious asset to the mucosal defence system. Finally, through their cytotoxic and secretory activities, NK cells may influence other classes of cells within microenviroments of the intestinal mucosa. It is proposed that an efficient suppressor cell system prevents excessive mucosal lymphoid proliferation in response to the sustained and variable antigenic challenge from the intestinal contents. The predominately suppressive role of NK cells in the immunoregulation of antibody and cell-mediated immune responses in the blood and primary lymphoid organs suggests that NK cells may perform similar functions in the intestine. Little is known however, about the mechanisms controlling intestinal epithelial or connective tissue cell growth, but cytotoxicity mediated by NK or LAK cells against these rapidly proliferating populations is one possible form of control. The capacity of peripheral blood NK cells to secrete multiple cytokines raises the possibility that similar or related cells may be responsible for the production of IL-2 or CSFs by intestinal lymphocytes.

A discussion of the possible functions of NK and LAK cells in the normal intestine leads to the hypothesis that abnormalities in the number or function of these cells may be of aetiological importance in diseases affecting the intestine. Critical to this experimental approach was the ability to isolate and assay populations of intestinal mucosal lymphocytes from a large number of cases of colon carcinoma, IBD and other nonmalignant conditions. The relevance of the mucosal nonspecific cytotoxic activity detected was reinforced by using epithelial cells and

fibroblasts derived from the intestinal mucosa as targets, in addition to standard tumour cell lines. In order to emphasize the advantages of using mucosal lymphoid tissue in the study of intestinal diseases, comparisons between mucosal and peripheral blood mononuclear cell populations were made when possible. In addition to functional assays of NK and LAK cell activity, the issue of mucosal nonspecific cytotoxicity was approached by the localizaton of intestinal NK cells by immunohistochemistry.

In order to postulate that NK and LAK cells exist in the intestine in vivo, it was also necessary to show that the intestinal mucosa is capable of sustaining these cells by the local production of cytokines. Therefore, lamina propria mononuclear cell populations (LPMC) were investigated for their ability to produce IL-2 and colony stimulating factor (CSF) activity in vitro. The investigation of mucosal CSF production may have little relevance to the function of NK and LAK cells, but it forms the basis for future studies of the role of the various CSF's in colon carcinoma and IBD.

9.2 Natural killer cells in the intestinal mucosa.

Assay of LPMC against K562 cells and epithelial tumour cells from the intestinal mucosa did not show substantial spontaneous or interferon (IFN)-augmented NK activity. When significant amounts of cytotoxic activity were detected in LPMC populations, the average levels of cytotoxicity were still markedly lower than those of peripheral blood mononuclear cells (PBMC). This paucity of NK activity is a strong

argument against a significant role for NK cell-mediated cytotoxicity in either the normal or diseased intestinal mucosa. The resistance of freshly isolated colon carcinoma cells to NK activity and the lack of an association between mucosal NK activity and the presence of colon cancer together imply that mucosal NK cells do not have an important antineoplastic function.

For similar reasons, the infrequency of NK cells in the mucosa of intestines affected by IBD suggests that NK cell activity is not involved in the tissue injury and inflammation which accompany this disorder. Antibody-dependent cellular cytotoxicity (ADCC) is another effector mechanism which was investigated in the intestinal mucosa. In the presence of antibodies directed against tumour cells or components of the normal intestine, NK cells or Tγ cells may participate in the pathogenesis of colon carcinoma or IBD. However, the very low levels of ADCC found in LPMC populations compared to the peripheral blood imply that NK cells and other cell types capable of ADCC are infrequent in the intestine and are therefore unlikely to mediate biologically significant ADCC even if the appropriate antibodies are present.

It could be argued that a small population of functionally important NK cells exist in the intestinal mucosa, but are obscured by the vast majority of LPMC which lack cytotoxic activity. Indeed, the function of this minority population may have been further adversely affected by suppressive factors, such as prostaglandins, released during their isolation. The studies presented in this thesis do not suggest that

suppressive factors or cells are a major factor responsible for the low levels of LPMC NK activity. However the data indicate that there is a minimal presence of NK cells in many resected intestinal specimens. The immunohistochemical survey using the Leu-7 antibody detected very occasional positive cells in the lamina propria which may have been NK cells, although it is known that T cells lacking NK activity may express this antigen. The finding of low level, but significant activity against K562 targets in a substantial proportion of specimens and the ability to boost this activity slightly using NK enrichment procedures and IFN treatment supports the existence of a minor, but variable intestinal NK cell presence. Whether these cells are represented in sufficient numbers to exert biologically relevant cytotoxic or immunoregulatory influences similar to those of peripheral blood NK cells is doubtful. At present, there is no immunohistological evidence to suggest that intestinal NK cells exert their effects by clustering in strategic microenviroments such as the subepithelial lamina propria or in and around colon carcinomas.

9.3 Lymphokine-activated killer cells in the intestinal mucosa.

The most important conclusion from the present studies was that the intestinal mucosa contains LAK precursor cells which can mature <u>in vitro</u> in response to IL-2. By inference, precursor cells may give rise to LAK effector cells within the intestinal mucosa. The identity of intestinal LAK cells was confirmed by their ability to lyse NK cell-resistant tumour

cell lines and freshly isolated colon carcinoma cells. Intestinal LAK cells could mediate ADCC, although they lacked the $CD16^+$ Fc receptors usually expressed by peripheral blood NK or LAK cells. Unlike NK cell or T cell-derived peripheral blood LAK cells, intestinal LAK cells reactive against K562 and colon cancer cells expressed a $CD2^+3^-8^-16^-$Leu-7^- phenotype. Analysis of intestinal LAK precursor cells showed a similar surface antigen profile. Recent studies of intestinal LAK precursor cells by other authors (321) provide partial confirmation of this phenotype except that the cells were $CD8^+$ and Leu-19^+. As yet, there have been no other phenotypic studies on intestinal LAK effector cells apart from those presented in this thesis. While the majority of peripheral blood LAK cells express the phenotype of their NK or T cell precursors, a minority population bears the $CD2^+3^-16^-$ Leu-19^+ phenotype which is similar to that of the intestinal LAK cell described here. The intestinal LAK precursor and effector cells characterized in this thesis were not examined for the Leu-19 antigen as the anti-Leu-19 monoclonal antibody was unavailable at the time of completion of these studies. Phenotypic studies of intestinal LAK cells mediating ADCC revealed that a small minority in some specimens were $CD16^+$ and therefore probably originated from infrequent NK cells within the LPMC populations. Thus, while the majority of intestinal LAK cells express a phenotype unrelated to any known cell lineage, a small proportion appear to resemble the most common type of peripheral blood LAK cell which is derived from IL-2-activated NK cells.

Conditions needed for the generation of intestinal LAK activity from precursor cells included an absolute requirement for IL-2 in concentrations sufficient to induce cellular proliferation. Inhibition of this process at the level of the IL-2 receptor using a monoclonal antibody directed against the receptor (Tac) or at the subsequent stage of cellular proliferation by mitomycin-C, blocked the induction of LAK activity. These data however, do not prove that IL-2 directly stimulates LAK cell precursors and they also do not exclude the possibility that additional cell types or cytokines such as IL-4 (297) may be involved in the generation of some forms of LAK cell. The induction of intestinal LAK activity was inhibited by physiological serum concentrations of hydrocortisone, unlike the proliferative response of activated T cells to exogenous IL-2 which was sensitive only to much higher hydrocortisone levels. This observation may indicate an intrinsic difference between the IL-2 receptor-mediated activation of T cells and LAK precursor cells.

Because studies of LAK cells describe a largely in vitro phenomenon, any extrapolations concerning their role in vivo must be speculative. The dependence of LAK activity on IL-2 suggests that LAK cells are most likely to exist in vivo as part of the amplification mechanisms arising from a specific immunological response. LAK cells are reactive against a much wider spectrum of tumour cells than NK cells and so may be an important part of the host's innate defences against malignant transformation. Like NK cells, LAK cells have a largely unexplored potential for cytotoxicity against microbial pathogens. Nothing is known about the

immunoregulatory properties of LAK cells, but they may exert control over immunological reactions by direct cytotoxicity and cytokine secretion. Therefore, the presence of LAK cells within the intestinal mucosa may confer protection against the development of malignancy, infection by microrganisms and excessive lymphoid proliferation in response to the antigen load of the intestinal contents.

Apart from the known potential of LAK activity against tumour cells in vitro, definitive evidence supporting an antineoplastic role for LAK cells in vivo is lacking. In the studies described in this thesis, the levels of LAK activity against K562 cells generated from histologically normal intestinal mucosa resected for benign or malignant conditions were comparable, suggesting that there was no in vivo abnormality of LAK precursor cell number or function associated with colon carcinoma. Moreover, comparable levels of LAK activity against colon carcinoma targets were generated from intestinal mucosae affected by cancer or by IBD and from the peripheral blood of normal subjects. This observation raises the possibility that the lysis of colon cancer cells by intestinal LAK cells reflects the broad, nonspecific cytotoxicity of the LAK cell phenomenon rather than a finding directly linking LAK cells to the pathogenesis of colon cancer.

The same arguments outlined for colon carcinoma apply when considering the role of intestinal LAK cells in IBD. Because of their ability to lyse fibroblasts and epithelial tumour cells from the intestine, LAK cells must be considered as possible mediators of mucosal injury and

inflammation in IBD. However there is, as yet, no evidence to indicate that intestinal LAK activity is abnormal in IBD. Moreover, the ability of intestinal LAK cells to lyse intestinal fibroblasts and epithelial cells is not restricted to IBD specimens and thus the relevance of this cytotoxic activity to the causation of IBD is questionable.

The place of LAK activity in the pathogenesis of colon carcinoma and IBD will not be settled until it is demonstrated that intestinal LAK effector cells can develop from their precursors in vivo in the intestinal mucosa. Mononuclear cells from the intestinal lamina propria have the capacity to produce IL-2 both spontaneously and after mitogen stimulation and the concentrations of IL-2 produced by mitogen-stimulated LPMC are of the same order of magnitude as the levels of recombinant IL-2 used to generate LAK cells in vitro. Therefore, conditions appear suitable for the activation of LAK precursor cells in the intestinal mucosa, but how these observations relate to the availability of IL-2 for LAK cell generation in the normal or diseased intestinal mucosa in vivo cannot be determined. Furthermore, although only IL-2 is apparently required for LAK cell generation in vitro, optimum requirements for the activation of LAK precursor cells in the intestinal mucosa may be more complex. The lower levels of IL-2 secreted spontaneously by LPMC indicates that under normal circumstances, LAK effector cells may not exist in large numbers in the intestinal mucosa. However, under conditions of immune stimulation which generate larger amounts of IL-2, LAK cells may represent an important multifunctional amplification mechanism which could have both

protective and deleterious effects.

LAK cells are able to function in vivo, as adoptive transfer studies in animals and man have established that culture-generated peripheral blood LAK cells have a clinically measurable antitumour effect in organs affected by malignancy. However, the studies reported in this thesis failed to find any indication of spontaneous LAK activity in freshly isolated LPMC populations. This finding may be artefactual as intestinal LAK cell activity may decline in the absence of IL-2 during the prolonged isolation procedure. In experimental animals, intestinal LAK cells have been reported following oral challenge with large doses of alloantigen. Moreover, in humans, peripheral blood lymphocytes with the cytotoxic properties of LAK cells have been reported in abnormal situations such as bone marrow transplantation, infectious mononucleosis and Tγ lymphoproliferative disease. Thus at present, the evidence supporting the existence of LAK cells in the peripheral blood of normal subjects or indeed, in the intestinal mucosa of patients with colon cancer or IBD is largely indirect.

9.4 Cytokine production by LPMC populations.

The ability of LPMC to produce cytotokines is an important consideration in the investigation of intestinal NK and LAK cell activity because without the local production of IL-2, it is unlikely that cells of this type exist or function in the intestinal mucosa. Therefore, the

production of significant amounts of IL-2 by mitogen-stimulated LPMC suggests that a favorable enviroment for NK and LAK cells may exist within the intestinal mucosa. Although there was a wide variation in the amounts of IL-2 produced by individual specimens, there was a statistically significant trend towards reduced levels of IL-2 production in association with colon carcinoma. This finding is consistent with reports of suppressive factors produced by colon cancers and raises the possibility that in vivo, mucosal defences such as NK or LAK cells are inhibited in the presence of carcinoma due to reduced availability of IL-2.

The cytokine(s) responsible for the CSF activity produced by LPMC were not identified in this thesis due to technical difficulties. Because the CSF activity measured may comprise several factors from different cell sources, the observation that total CSF activity was unaffected by the presence of cancer is of questionable significance. Nevertheless, the demonstration of endogenous CSF production in the intestinal mucosa is highly relevant to the function of resident intestinal macrophages and to granulocytes which migrate into the mucosa in response to inflammatory stimuli.

The production of IL-2 and CSF activity by LPMC indicates that one or more cell types with important immunoregulatory potential exist in the intestinal lamina propria. The identity of the producer cells was not established, but it is possible that the cytokine activity assayed may have been derived from several different cell types. Although NK cells or related large granular lymphocytes secrete IL-2 and CSF's, these cells

were not present in the intestinal mucosa in sufficient numbers to account for the amounts of cytokine assayed.

9.5 Directions for future research.

Consideration of the data presented and discussed here suggests further areas of investigation which address questions left unanswered by this thesis. One of the main issues raised in this thesis is whether a small minority of cells possessing NK or LAK activity exists in the human intestinal mucosa. Evidence presented here and elsewhere supports the existence of small numbers of mucosal NK cells, but methods used to detect and characterize them have so far been too imprecise. The isolation of homogenous minority cell populations from the intestinal mucosa would best be achieved by fluorescent-activated cell sorting or cell panning using selected monoclonal antibodies. The surface antigens of most interest would be those found on peripheral blood NK and LAK cells (CD7, CD16, Leu-19 and LAK-1). Populations of LPMC selected on the basis of expression of a particular surface antigen could then be tested for spontaneous cytotoxicity against a panel of targets sensitive to NK and LAK cells. The targets used should include freshly isolated colon carcinoma cells and intestinal epithelial cells, in addition to established tumour cell lines of known susceptibility. Further studies which could be carried out include assay of spontaneous cytotoxicity after short term (up to 18 h) incubation with IL-2 and IFN, in order to detect the full potential

of any cytotoxic cells present. The LPMC populations can also be assessed for their potential as LAK precursor cells by culture with IL-2 and IL-4 for longer periods (2-6 days). Characterization of the surface antigen profile expressed on generated LAK cells can then be performed.

The isolation of purified LPMC expressing uncommon surface antigens requires a large starting population of LPMC (approximately 10^9 cells). This number of cells is obtainable with the isolation techniques used in this thesis. Parallel experiments using peripheral blood NK and LAK cells would be needed to control for the possible inhibitory or stimulatory effects of the monoclonal antibodies on LPMC cytotoxicity.

Another issue needing investigation is the identification of surface epitopes unique to peripheral blood or intestinal LAK cells. The preparation of monoclonal antibodies specific for these antigens would allow the detection of LAK cells in normal and pathological situations and facilitate their isolation for further characterization. This strategy would be the most effective way of determining whether LAK effector cells exist in vivo. In order to raise monoclonal antibodies against LAK cells, purified or preferably cloned intestinal LAK cell populations could be used as the immunogen in standard monoclonal antibody preparation protocols. Such an approach has been used recently to prepare the LAK-1 monoclonal antibody which is reactive with both peripheral blood LAK precursor and effector cells (316).

The field of cytokine production by intestinal lymphocytes is of potentially great importance in elucidating the pathogenesis of malignant

and chronic inflammatory diseases of the human intestine. The cytokines described in this thesis require further characterization and identification of the cell(s) of origin. The T cell growth factor produced by LPMC is likely to consist mainly of IL-2, but a contribution from IL-4 is possible. The effect of recombinant IL-4 on the IL-2 assay used in this thesis is the first line of investigation to pursue. If the CTTL line used is responsive to IL-4, then the supernatants of mitogen-stimulated LPMC can be probed for IL-4 by using ion exchange chromatography and specific inhibitory antisera. The use of hydrophobic interaction chromatography and specific inhibitory antisera will also determine whether the CSF activity produced by LPMC is due to the presence of G-CSF, GM-CSF, IL-3 or other CSF's. Investigating the amount and types of cytokine produced by purified or cloned LPMC subpopulations will assist in identifying the cells responsible for cytokine production in the intestinal mucosa.

The intestinal mucosa provides an ideal enviroment in which to study the biology of cytokines associated with antigen presentation and antibody class switching. The production of Interleukin 1 in the intestinal mucosa has not been studied and the question as to whether antigen presenting cells such as intestinal macrophages, epithelial cells or dendritic cells produce this cytokine is also unanswered (502). Little is known about the production and properties of immunoglobulin class switch factors in the human intestine. Recent evidence indicates that the switching of IgM^+ B cells to $IgG_1{}^+$ or IgE^+ cells requires the participation of IL-4 (471). IL-4 may also enhance antigen presentation by activating

macrophages and increasing their expression of Ia molecules (503). Mucosal IL-4 production may thus be critical to the functioning of the secretory immune system and it follows that cells capable of secreting this cytokine and switch factors for IgA are likely to exist in the intestinal mucosa. LPMC populations isolated from the small and large bowel would therfore be a logical population to probe for the production of these cytokines.

9.6 Conclusion

The main conclusions of this thesis form the basis for further investigations into the pathogenesis of the important human diseases colon carcinoma and IBD. On numerical and functional grounds, NK cells probably exist in the intestine in small numbers, but their cytotoxic activity is unlikely to be an important factor in the biology of these diseases. However, plentiful numbers of LAK precursor cells with the potential to give rise to highly cytotoxic LAK effector cells are found within the intestinal mucosa in both colon carcinoma and IBD. The ability of LPMC to produce concentrations of IL-2 appropriate for the generation of intestinal LAK cells suggest that these cells exist within the intestinal mucosa. Further investigation is needed to determine the extent and relevance of the LAK cell phenomenon in vivo.

REFERENCES.

1. Herberman RB, Nunn ME, Lavrin DH, Asofsky R. Effect of antibody to θ antigen on cell-mediated immunity induced in syngeneic mice by murine sarcoma virus. J Nat Cancer Inst 1973;51:1509-12.
2. Hellstrom I, Hellstrom KE, Sjogren HO, Warner G. Demonstration of cell-mediated immunity to human neoplasms of various histological types. Int J Cancer 1971;7:1-16.
3. Takasugi M, Mickey MR, Terasaki PJ. Reactivity of lymphocytes from normal persons on cultured cells. Cancer Res 1973;33:2898-2902.
4. Jondal M, Pross H. Surface markers on human B and T lymphocytes VI. Cytotoxicity against cell lines as a functional marker for lymphocyte populations. Int J Cancer 1975,15:596-605.
5. Ortaldo JR,Herberman RB. Heterogeneity of natural killer cells. Ann Rev Immunol 1984;2:359-94.
6. Andersson LC, Nilsson K, Gahmberg CG. K562-a human erthroleukaemic cell line. Int J Cancer 1979;23:143-7.
7. Minowada J, Ohnuma T, Moore GE. Rosette-forming human lymphoid cell lines : I. Establishment and evidence for origin of thymus-derived lymphocytes. J Nat Cancer Inst 1972;49:891-5.
8. Pattengale PK, Gidlund M, Nilsson K, Sundstrom C, Orn A, Wigzell H. Lysis of human B-lymphocyte-derived lymphoma/leukaemia cells of established cell lines by interferon-activated natural killer (NK) cells. Int J Cancer 1981;28:459-68.

9. Hersey P, Edwards A, Honeyman M, McCarthy WH. Low natural killer cell activity in familial melanoma patients and their relatives. Br J Cancer 1979;40:113-22.

10. Cannon GB, Bonnard GD, Djeu J, West WH, Herberman RB. Relationship of human natural lymphocyte-mediated cytotoxicity of breast-cancer-derived target cells. Int J Cancer 1977;19:487-97.

11. Grimm EA, Mazumder A, Zhang HZ, Rosenberg SA. Lymphokine-activated killer cell phenomenon. Lysis of natural killer resistant fresh solid tumor cells by interleukin 2-activated autologous human peripheral blood lymphocytes. J Exp Med 1982;155:1823-41.

12. Oshimi K, Oshimi Y, Yamada O, Mizoguchi H. Lysis of lymphoma cellls by autologous and allogeneic natural killer cells. Blood 1985;65:638-43.

13. Uchida A, Micksche M. Lysis of fresh human tumour cells by autologous large granular lymphocytes from peripheral blood and pleural effusions. Int J Cancer 1983;32:37-44.

14. Lotzova E, Savary CA. Generation of NK cell activity from human bone marrow. J Immunol 1987;139:279-84.

15. Timonen T, Ortaldo JR, Herberman RB. Characteristics of human large granular lymphocytes and relationship to natural killer and K cells. J Exp Med 1981;153:569-82.

16. Timonen T, Ortaldo JR, Herberman RB. Analysis by a single cell cytotoxicity assay of natural killer (NK) cell frequencies among human large granular lymphocytes and the effects of interferon on their activity. J Immunol 1982;128:2514-21.

17. Lanier LL, Kipps TJ, Phillips JH. Functional properties of a unique subset of cytotoxic $CD3^+$ T lymphocytes that express Fc receptors for IgG (CD16/Leu-11 antigen). J Exp Med 1985;162:2089-2106.

18. Grossi CE, Ferrarini M. Morphology and cytochemistry of human large granular lymphocytes. In: Herberman RB (ed) NK cells and other natural effector cells, Academic Press, New York,1982, 1-8.

19. Ferrarini M, Gross CE. Ultrastructure and cytochemistry of the human large granular lymphocytes. In: Lotzova E, Herberman RB (eds) Immunobiology of Natural Killer Cells, CRC Press Inc., Florida, Vol I,1986,33-43.

20. Abo T, Miller CA, Balch CM. Characterization of granular lymphocyte subpopulations expressing HNK-1 (leu-7) and Leu-11 antigens in the blood and lymphoid tissues from fetuses, neonates and adults. Eur J Immunol 1984;14:616-23.

21. Eggen BM. Cytotoxicity of temporarily adherent human mononuclear blood cells in monocyte monolayers. Int Arch Allergy Appl Immunol 1983;72:143-9.

22. Botazza B, Introna M, Allavena P, Villa A, Mantovani A. In vitro migration of human large granular lymphocytes. J Immunol 1985;134:2316-21.

23. Zoller M, Bellgrau D, Axberg I, Wigzell H. Natural killer cells do not belong to the recirculating lymphocyte population.
Scan J Immunol 1982;15:159-167.

24. Oldham RK, Forbes JT, Neblack GD. Natural killer activity of human thoracic duct lymphocytes (abstr).
Proc Amer Assoc Cancer Res 1978;19:161.

25. Timonen T, Reynolds CW, Ortaldo JR, Herberman RB. Isolation of human and rat natural killer cells.
J Immunol Methods 1982;51:269-77.

26. Weissler JC, Nicod LP, Lipscomb MF, Toews GB. Natural killer cell function in human lungs is compartmentalized.
Amer Rev Resp Dis 1987;135:941-9.

27. Hogan PG, Hapel AJ, Doe WF. Lymphokine-activated and natural killer cell activity in human intestinal mucosa.
J Immunol 1986;135:1731-8.

28. Lanier LL, Engelman EG, Gatenby P, Babcock GF, Warner NL, Herzenberg LA. Correlation of functional properties of human lymphoid cell subsets and surface marker phenotypes using multiparameter analysis and flow cytometry.
Immunol Rev 1983;74:143-60.

29. Lanier LL, Le AM, Civin CI, Loken WR, Phillips JH. The relationship of CD16 (leu-11) and Leu-19 (NKH-1) antigen expression on human peripheral blood NK cells and cytotoxic T lymphocytes.
J Immunol 1986;136:4480-6.

30. Schmidt RE, Murray C, Daley JF, Schlossman SF, Ritz J. A subset of natural killer cells in peripheral blood displays a mature T cell phenotype. J Exp Med 1986;164:351-6.

31. Fitzgerald PA, Evans R, Kirkpatrick D, Lopez C. Heterogeneity of human NK cells: comparison of effectors that lyse HSV-1-infected fibroblasts and K562 erythroleukaemia targets. J Immunol 1983;131:1663-7.

32. Phillips WH, Ortaldo JR, Herberman RB. Selective depletion of human natural killer cells on monolayers of target cells. J Immunol 1980;125:2322-7.

33. Krensky AM, Ault KA, Reiss CS, Strominger JL, Burakoff SJ. Generation of long-term cytolytic cell lines with persistent natural killer cell activity. J Immunol 1982;129:1748-51.

34. Zarling JM, Kung PC. Monoclonal antibodies distinguish between human NK cells and cytotoxic T lymphocytes. Nature 1980;288:394-6.

35. Perussia B, Fanning V, Trinchieri G. A human NK and K subset shares with cytotoxic T cells expression of the antigen recognized by antibody OKT8. J Immunol 1983;131:223-31.

36. Allavena P, Ortaldo JR. Separation and characterization of phenotypically distinct subsets of NK cells. In: Lotzova E, Herberman RB (eds) Immunobiology of Natural Killer Cells, CRC Press Inc, Florida, Vol I,1986,21-32.

37. Griffin JD, Hercend T, Beveridge R, Schlossman S. Characterization of an antigen expressed by human killer cells.
J Immunol 1983;130:2947-51.

38. Ortaldo JR, Mason A, Overton R. Lymphokine-activated killer cells. Analysis of progenitors and effectors.
J Exp Med 1986;164:1193-1205.

39. Lanier LL, Phillips JH, Hackett J, Tutt M, Kumar V. Natural killer cells: definition of a cell type rather than a function.
J Immunol 1986;137:2735-9.

40. Abo T, Cooper MD, Balch CM. Characterization of HNK-1^+ (Leu-7) human lymphocytes I. Two distinct phenotypes of human NK cells with different cytotoxic capability. J Immunol 1982;129:1752-7.

41. Lanier LL, Cwirla S, Phillips JH. Genomic organization of γ genes in human peripheral blood natural killer cells.
J Immunol 1986;137: 3375-7.

42. Lanier LL, Ruitenberg JJ, Phillips JH. Human $CD3^+$ lymphocytes express neither CD4 nor CD8 antigens. J Exp Med 1986;164:339-344.

43. Borst J, van de Griend RJ, van Oostveen JW, Ang SL, Melief CJ, Seidman JG, Bolhuis RLH. A T-cell receptor γ/CD3 complex found on cloned functional lymphocytes. Nature 1987;325: 683-8.

44. Muchmore AV. A comparison of antibody-dependent cellular cytotoxicity (ADCC) and NK activity. In: Lotzova E, Herberman RB (eds) Immunobiology of Natural Killer Cells, CRC Press Inc, Florida,1986, Vol II, 201-213.

45. Sokol RJ, Hewitt S. Autoimmune haemolysis: a critical review. CRC Critical Reviews in Haematology/Oncology 1985;4:125-54.

46. Rook AH, Lane HC, Folks T, McCoy S, Alter H, Fauci A. Sera from HTLV-III/LAV antibody-positive individuals mediate antibody-dependent cellular cytotoxicity against HTLV III/LAV-infected T cells. J Immunol 1987;138:1064-7.

47. Tagliabue A, Nencioni L, Villa L, Keren DF, Lowell GH, Boraschi D. Antibody-dependent cell-mediated antibacterial activity of intestinal lymphocytes with secretory IgA. Nature 1983;306:184-6.

48. Kanwar SS, Ganguly NK, Walia BNS, Mahajan RC. Direct and antibody dependent cell mediated cytotoxicity against Giardia lamblia by splenic and intestinal lymphoid cells in mice. Gut 1986;27:73-7.

49. Diamond RD. Antibody-dependent killing of Cryptococcus neoformans by human peripheral blood natural killer cells. Nature 1974;247:148-50.

50. Bradley TP, Bonavida B. Mechanism of cell-mediated cytotoxicity at the single cell level IV. Natural killing and antibody-dependent cellular cytotoxicity can be mediated by the same human effector cell as determined by the two-target conjugate assay. J Immunol 1982;129:2260-5.

51. Allavena P, Ortaldo JR. Characteristics of human NK clones: target specificity and phenotype. J Immunol 1984;132:2363-9.

52. Merrill JE. Natural killer (NK) and antibody-dependent cellular cytotoxicity (ADCC) activities can be differentiated by their different seneitivities to interferon and prostaglandin E_1.
J Clin Immunol 1983;3:42-50.

53. Kay HD, Bonnard GD, West WH, Herberman RB. A functional comparison of human Fc-receptor-bearing lymphocytes active in natural cytotoxicity and antibody-dependent cellular cytotoxicity.
J Immunol 1977;118:2058-66.

54. Katz JD, Mitsuyasu R, Gotlieb MS, Lebow LT, Bonavida B. Mechanism of defective NK cell activity in patients with acquired immunodeficiency syndrome (AIDS) and AIDS-related complex II. Normal antibody-dependent cellular cytotoxicity (ADCC) mediated by effector cells defective in natural killer (NK) cytotoxicity.
J Immunol 1987;139:55-60.

55. Wahlin B, Perlmann P. Characterization of human K cells by surface antigens and morphology at the single cell level.
J Immunol 131;2340-47.

56. Abo T, Miller CA, Gartland GL, Balch CM. Differentiation stages of human natural killer cells in lymphoid tissues from fetal to adult life. J Exp Med 1983;157:273-84.

57. Savary CA, Lotzova E. Phylogeny and ontogeny of NK cells. In: Lotzova E, Herberman RB (eds) Immunobiology of Natural Killer Cells, CRC Press Inc, Florida, 1986, Vol I, 45-61.

58. Springer TA, Dustin ML, Kishimoto TK, Marlin SD. The lymphocyte function-associated LFA-1, CD2, and LFA-3 molecules: cell adhesion receptors of the immune system. Ann Rev Immunol 1987;5:223-52.

59. Peter HH. The origin of human NK cells. An ontogenic model derived from studies in patients with immunodeficiencies. Blut 1983;46:239-48.

60. Phillips JH, Lanier LL. A model for the differentiation of human natural killer cells. Studies on the in vitro activation of Leu-11$^+$ granular lymphocytes with a natural killer-sensitive tumour cell, K562. J Exp Med 1985;161:1464-82.

61. Perussia B, Starr S, Abraham S, Fanning V, Trinchieri G. Human natural killer cells analysed by B73.1, a monoclonal antibody blocking Fc receptor functions. J Immunol 1983;130:2133-41.

62. Seki H, Ueno Y, Taga K, Matsuda A, Mikawaki T, Taniguchi N. Mode of in vitro augmentation of natural killer cell activity by recombinant human interleukin 2: a comparative study of Leu-11$^+$ and Leu-11$^-$ cell populations in cord blood and adult peripheral blood. J Immunol 1985;135:2351-6.

63. Haller O, Kiessling R, Orn A, Wigzell H. Generation of natural killer cells: an autonomous function of the bone marrow. J Exp Med 1977;145:1411-6.

64. Haller O, Wigzell H. Suppression of natural killer cell activity with radioactive strontium: effector cells are marrow dependent. J Immunol 1977:118:1503-6.

65. Rooney CM, Wimperis JZ, Brenner MK, Patterson J, Hoffbrand AV, Prentice HG. Natural killer cell activity following T cell-depleted allogeneic bone marrow transplantation. Brit J Haem 1986;62:413-20.

66. Spina C. Azathioprine as an immune modulating drug: clinical implications. In: Mitchell MS, Fahey JL (eds) Immune suppression and modulation, Clinics in immunology and allergy, WB Saunders, Philadelphia,1984, 415-46.

67. Ueno Y, Miyawaki T, Seki H, Matsuda A, Taga K, Sato H, Taniguchi N. Differential effects of recombinant human interferon-γ and interleukin 2 on natural killer cell activity of peripheral blood in early human development. J Immunol 1985;135:180-4.

68. Trinchieri G, Matsumoto-Kobayashi M, Clark SC, Seehra J, London L, Perussia B. Response of resting human peripheral blood natural killer cells to interleuukin 2. J Exp Med 1984;160:1147-69.

69. Itoh K, Shiba K, Shimizu Y, Suzuki R, Kumagai K. Generation of activated killer (AK) cells by recombinant interleukin 2 (rIL 2) in collaboration with interferon-γ (IFN-γ). J Immunol 1985;134:3124-9.

70. Ortaldo JR, Mason AT, Gerard JP, Henderson LE, Farrar W, Hopkins RF, Herberman RB, Rabin H. Effects of natural and recombinant IL 2 on reguation of IFN-γ production and natural killer cell activity: lack of involvement of the Tac antigen for these immunoregulatory effects. J Immunol 1984;133:779-83.

71. Domzig W, Stadler BM, Herberman RB. Interleukin 2 dependence of human natural killer (NK) cell activity. J Immunol 1983;130:1970-3.

72. Lanier LL, Benike CJ, Phillips JH, Engeleman EG. Recombinant interleukin 2 enhanced natural killer cell-mediated cytotoxicity in human lymphocyte subpopulations expressing the Leu 7 and Leu 11 antigens. J Immunol 1985;134:794-801.

73. Smith KA. The two chain structure of high affinity IL-2 receptors. Immunology Today 1987;8:11-13.

74. Miyawaki T, Yachie A, Uwadana N, Ohzeki S, Nagaoki T, Taniguchi N. Functional significance of Tac antigen expressed on activated human T lymphocytes: Tac antigen interacts with T cell growth factor in cellular proliferation. J Immunol 1982;129:2474-8.

75. Vose B, Bonnard GD. Limiting dilution analysis of the frequency of human T cells and large granular lymphocytes proliferating in response to interleukin 2 I. The effect of lectin on the proliferative frequency and cytolytic activity of cultured lymphoid cells. J Immunol 1983;130:687-93.

76. Vose BM, Riccardi C, Bonnard GD, Herberman RB. Limiting dilution analysis of the frequency of human T cells and large granular lymphocytes proliferating in response to interleukin 2 II. Regulatory role of interferon on proliferative and cytotoxic precursors. J Immunol 1983;130:768-72.

77. Suzuki R, Handa K, Itoh K, Kumagai K. Natural killer cells as a responder to interleukin 2 (IL 2) I. Proliferative response and establishment of cloned cells. J Immunol 1983;130:981-7.

78. London L, Perussia B, Trinchieri G. Induction of proliferation in vitro of resting human natural killer cells: expression of surface activation antigens. J Immunol 1985;134:718-27.

79. Itoh K, Tilden AA, Kumagai K, Balch CM. Leu-11$^+$ lymphocytes with natural killer (NK) activity are precursors of recombinant interleukin 2 (rIL 2)-induced activated killer (AK) cells. J Immunol 1985;134:802-7.

80. Ben Aribia MH, Leroy E, Lantz O, Metivier D, Autran B, Charpentier B, Hercend T, Senik A. rIL 2-induced proliferation of human circulating NK cells and T lymphocytes: synergistic effects of IL 1 and IL 2. J Immunol 1987;139:443-51.

81. Shirakawa F, Tanaka Y, Eto S, Suzuki H, Yodoi J, Yamashita U. Effect of interleukin 1 on the expression of interleukin 2 receptor (Tac antigen) on human natural killer cells and natural killer-like cell line (YT cells). J Immunol 1986;137:551-6.

82. Ostensen ME, Thiele DL, Lipsky PE. Tumor necrosis factor-α enhances cytolytic activity of human natural killer cells. J Immunol 1987;138:4185-91.

83. Mannering GJ, Deloria LB. The pharmacology and toxicology of the interferons: an overview. Ann Rev Pharmacol Toxicol 1986;26:455-515.

84. Handa K, Suzuki R, Matsui H, Shimizu Y, Kumagai K. Natural killer (NK) cells as a responder to interleukin 2 (IL 2) II. IL 2-induced interferon γ production. J Immunol 1983;130:988-92.

85. Ortaldo JR, Herberman RB. Augmentation of natural killer activity. In: Lotzova E, Herberman RB (eds) Immunobiology of Natural Killer cells, CRC Press Inc, Florida, Vol II, 1986:145-162.

86. Wright SC, Bonavida B. Studies on the mechanism of natural killer (NK) cell-mediated cytotoxicity. III. Interferon-pretreatment of effector cell augments the lytic activity of natural killer cytotoxic factors (NKCF). J Immunol 1983;130:2960-4.

87. Moore M, Kimber I. Augmentation of human NK and ADCC by interferon. In: Herberman RB (ed) Natural Cell-Mediated Immunity Against Tumours, Academic Press, New York,1980, 569-79.

88. Pattengale PK, Sundstrom C, Yu AL, Levine A. Lysis of fresh leukaemic blasts by interferon-activated human natural killer cells. Nat Immun Cell Growth Regul 1983/84;3:165-80.

89. Wright SC, Bonavida B. Studies on the mechanism of natural killer (NK) cell-mediated cytotoxicity. IV. Interferon-induced inhibition of NK target cell susceptibility is due to a defect in their ability to stimulate release of natural killer cytotoxic factors (NKCF). J Immunol 1983;130:2965-8.

90. Ortaldo JR, Phillips W, Wasserman K, Herberman RB. Effects of metabolic inhibitors on spontaneous and interferon-boosted natural killer cell activity. J Immunol 1980;125:1839-44.

91. Droller MJ, Schneider MU, Perlmann P. A possible role of prostaglandins in the inhibition of natural and antibody-dependent cell-mediated cytotoxicity against tumour cells.
Cell Immunol 1978;39:165-77.

92. Droller MJ, Perlmann P, Schneider MU. Enhancement of natural and antibody-dependent lymphocyte cytotoxicity by drugs which inhibit prostaglandin production by tumor target cells.
Cell Immunol 1978;39:154-64.

93. Brunda MJ, Herberman RB, Holden HT. Inhibition of murine natural killer cell activity by prostaglandins. J Immunol 1980;124:2682-90.

94. Goto T, Herberman RB, Maluish A, Strong DM. Cyclic AMP as a mediator of prostaglandin E-induced suppression of human natural killer cell activity. J Immunol 1982;130:1350-5.

95. Weissman DN, de Shazo RD, Banks DE. Modulation of natural killer cell function by human alveolar macrophages.
J Allergy Clin Immunol 1986;78:571-7.

96. Bordignon C, Allavena P, Introna M, Biondi A, Bottazzi B, Mantovani A. Modulation of NK activity by human mononuclear phagocytes: suppressive activity of broncho-alveolar macrophages. In: Herbermen RB (ed) NK cells and Other Natural Effector Cells, Academic Press, New York, 1982, 581-8.

97. Introna M, Allavena P, Acero R, Colombo N, Molina P, Mantovani A. Natural killer activity in human ovarian tumours. In; Herbermen RB (ed) NK cells and Other Natural Effector Cells, Academic Press, New York, 1982, 1119-26.

98. Cunningham-Rundles S. Control of natural cytotoxicity in the regional lymph node in breast cancer. In: Herbermen RB (ed) NK cells and other natural effector cells, Academic Press, New York, 1982, 1133-40.

99. Leung KH, Koren HS. Regulation of human natural killing II. Protective effect of interferon on NK cells from suppression by PGE_2. J Immunol 1982;129:1742-7.

100. D'Amore PJ, Golub SH. Suppression of human NK cell cytotoxicity by an MLC-generated cell population. J Immunol 1985;134:272-9.

101. Nair MPN, Schwartz SA. Suppression of human natural and antibody-dependent cytotoxicity by soluble factors from unstimulated normal lymphocytes. J Immunol 1982;129:2511-8.

102. Dempsey RA, Dinarello CA, Mier JW, Rosenwasser LJ, Allegretta M, Brown TE, Parkinson DR. The differential effects of human leukocyte pyrogen/lymphocyte activating factor, T cell growth factor, and interferon on human natural killer activity. J Immunol 1982;129:2504-10.

103. Herman J, Dinarello CA, Kew MC, Rabson AR. The role of interleukin 1 (IL 1) in tumour-NK cell interactions: correction of defective NK cell activity in cancer patients by treating target cells with IL 1. J Immunol 1985;135:2882-6.

104. Rotrosen D, Gallin JI. Disorders of phagocyte function. Ann Rev Immunol 1987;5:127-50.

105. El-Hag A, Clark RA. Down-regulation of human natural killer activity against tumours by the neutrophil myeloperoxidase system and hydrogen peroxide. J Immunol 1984;133:3291-7.

106. Hanna N, Schneider M. Enhancement of tumour metastasis and suppression of natural killer cell activity by β-estradiol treatment. J Immunol 1983;130:974-80.

107. Baley JE, Schacter BZ. Mechanisms of diminished natural killer cell activity in pregnant women and neonates. J Immunol 1985;134:3042-8.

108. Hersey P. The evolving role of alpha interferon in the treatment of malignancies. Aust NZ J Med 1986;16:425-37.

109. Golub SH, Moy PH. In vivo modulation of NK activity in cancer patients. In: Lotzova E, Herberman RB (eds) Immunobiology of Natural Killer Cells, CRC Press Inc, Florida, 1986, Vol II, 56-74.

110. Golomb HM. The treatment of Hairy Cell Leukaemia. Blood 1987;69:979-83.

111. Rosenberg SA, Lotze MT, Muul LM, Chang AE, Avis FP, Leitman S, Linehan WM, Robertson CN, Lee RE, Rubin JT, Seipp CA, Simpson CG, White DE. A progress report on the treatment of 157 patients with advanced cancer using lymphokine-activated killer cells and interleukin-2 or high dose interleukin-2 alone. New Eng J Med 1987;316:889-97.

112. Lotze MT, Frana LW, Sharrow SO, Robb RJ, Rosenberg SA. In vivo administration of purified human interleukin-2 I. Half life and immunological effects of the Jurkat cell line-derived interleukin 2. J Immunol 1985;134:157-66.

113. Wiltrout RH, Mathieson BJ, Talmadge JE, Reynolds CW, Zhang SR, Herberman RB, Ortaldo JR. Augmentation of organ-associated natural killer activity by biological response modifiers. Isolation and characterization of large granular lymphocytes from the liver. J Exp Med 160:1431-49.

114. West WH, Tauer KW, Yanneli JR, Marshall GD, Orr DW, Thurman GB, Oldham RK. Constant infusion recombinant interleukin-2 adoptive immunotherapy of advanced cancer. New Eng J Med 1987;316:898-905.

115. Hiserodt JC, Britvan LJ, Targan SR. Characterization of the cytolytic reaction mechanism of the human natural killer (NK) lymphocyte : resolution into binding, programming, and killer cell-independant steps. J Immunol 1982;129:1782-7.

116. Hiserodt JC, Britvan LJ, Targan SR. Differential effects of various pharmacological agents on the cytolytic reaction mechanism of the human natural killer lymphocyte : further resolution of programming for lysis and KCIL into discrete stages. J Immunol 1982;129:2266-70.

117. Ades EW, Hinson A. Effector cell sensitivity to sugar moities: inhibition of human natural killer cell activity by tunicamycin. Cell Immunol 1982;72:326-31.

118. Roder JC, Kiessling R. Target-effector interaction in the natural killer cell system I. Covariance and genetic control of cytolytic and target cell-binding subpopulations in the mouse. Scand J Immunol 1978;8:135-44.

119. Perussia B, Acuto O, Terhorst C, Faust JF, Lazarus R, Fanning V, Trinchieri G. Human natural killer cells analysed by B73.1, a monoclonal antibody blocking Fc receptor functions II. Studies of B73.1 antibody-antigen interaction on the lymphocyte membrane. J Immunol 1983;130:2142-8.

120. Obexer G, Rumpold H, Kraft D. Target-effector-cell interactions in the human natural-killer system: isolation of target structures. Immunobiol 1983;165:15-26.

121. Roder JC, Ahrlund-Richter L, Jondel M. Target-effector interaction in the human and murine natural killer system. Specificity and xenogeneic reactivity of the solubilized natural killer-target structure complex and its loss in a somatic cell hybrid. J Exp Med 1979;150:471-81.

122. Werkmeister JA, Roder JC, Curry C, Pross H. The effect of unphosphorylated and phophorylated sugar moieties on human and mouse natural killer cell activity: is there selsective inhibition at the level of target recognition and lytic acceptor site? Cell Immunol 1983;80:172-86.

123. Vodenlich L, Sutherland R, Schneider C, Newman RA, Greaves M. Receptor for transferrin may be a "target" structure for natural killer cells. Proc Natl Acad Sci USA 1983;80:835-9.

124. Newman RA, Warner JF, Dennert G. NK recognition of target structures: is the transferrin receptor the NK target structure? J Immunol 1984;133:1841-5.

125. Bridges KR, Smith BR. Discordance between transferrin receptor expression and susceptibility to lysis by natural killer cells. J Clin Invest 1984;76:913-8.

126. Alarcon B, Fresno M. Specific effect of anti-transferrin antibodies on natural killer cells directed against tumor cells. Evidence for the transferrin receptor being one of the target structures recognized by NK cells. J Immunol 1985;134:1286-91.

127. Trowbridge IS, Omary MB. Human cell surface glycoptotein related to cell proliferation is the receptor for transferrin. Proc Natl Acad Sci USA 1981;78:3039-43.

128. Sutherland R, Delia D, Schneider C, Newman R, Kemshead J, Greaves M. Ubiquitous cell-surface glycoprotein on tumor cells is proliferation-associated receptor for transferrin. Proc Natl Acad Sci USA 1981;78:4515-9.

129. Bishop GA, Glorioso JC, Schwartz SA. Relationship between expression of Herpes simplex virus glycoproteins and susceptibility of target cells to human natural killer cell activity. J Exp Med 1983;157:1544-61.

130. Werkmeister JA, Helfand SL, Haliotis T, Pross HF, Roder JC. The effect of target cell differentiation on human natural killer cell activity: a specific defect in target cell binding and early activation events. J Immunol 1982;129:413-8.

131. Harris JF, Chin J, Jewett MA, Kennedy M, Gorczynski RM. Monoclonal antibodies against SSEA-1 antigen: binding properties and inhibition of human natural killer cell activity against target cells bearing SSEA-1 antigen. J Immunol 1984;132:2502-9.

132. Werkmeister JA, Pross HF, Roder JC. Modulation of K562 cells with sodium butyrate. Association of impaired NK susceptibility with sialic acid and analysis of other parameters. Int J Cancer 1983;32:71-8.

133. Storkus WJ, Howell DN, Salt RD, Dawson JR, Cresswell P. NK susceptibility varies inversely with target cell class I HLA antigen expression. J Immunol 1987;138:1657-9.

134. Kornbluth J, Spear B, Raab SS, Wilson DB. Evidence for the role of class I and class II HLA antigens in the lytic function of a cloned line of human natural killer cells. J Immunol 1985;134:728-35.

135. Vargas-Cortes M, Hellstrom U, Perlmann P. Surface markers of human natural killer cells as analysed in a modified single cell cytotoxicity assay on poly-L-lysine coated cover slips. J Immunol Methods 1983;62:87-99.

136. Kohl S, Springer TA, Schmalsteig FC, Loo LS, Anderson DC. Defective natural killer cytotoxicity and polymorphonuclear leukocyte antibody-dependent cellular cytotoxicity in patients with LFA-1/OKM-1 deficiency. J Immunol 1984;133:2972-8.

137. Uggla CK, Jondal M, Kaplan D, Flomberg N, Knowles RW. Enhancement of natural killer cell activity by unique antibodies within the CD2 (sheep-RBC receptor) and CD16 (Fc receptor) clusters. In: McMichael AJ (ed), Leucocyte Typing III White Cell Differentiation Antigens, Oxford University Press, Oxford, 1987, 134-7.

138. Targan SR, Newman W. Definition of a "trigger" stage in the NK cytolytic reaction sequence by a monoclonal antibody to the glycoprotein T-200. J Immunol 1983;131:1149-53.

139. Sparrow RL, McKenzie IFC. A function for human T200 in natural killer cytolysis. Transplantation 1983;36:166-71.

140. Hoffman T, Herata F, Bougnox P, Fraser BA, Goldfarb RH, Herberman RB, Axelrod J. Phospholipid methylation and phospolipase A_2 activation in cytotoxicity by human natural killer cells. Proc Natl Acad Sci USA 1981;78:3839-43.

141. Hirata F, Axelrod J. Enzymatic methylation of phosphatidylethanolamine increases erythrocyte membrane fluidity. Nature 1978;275:219-20.

142. Quan PC, Ishizaka T, Bloom BR. Studies on the mechanism of NK cell lysis. J Immunol 1982;128:1786-91.

143. Hiserodt JC, Britvan LJ, Targan SR. Inhibition of human natural killing by heterologous and monoclonal antibodies. J Immunol 1982;129:2248-54.

144. Moon TD, Morley JE, Vessella RL, Lange PH. The role of calmodulin in human natural killer cell activity. Scand J Immunol 1983;18:255-58.

145. Weissman G, Goldstein I, Hoffstein S, Tsung PK. Reciprocal effects of cAMP and cGMP on microtubule-dependent release of lysosomal enzymes. Ann N Y Acad Science 1975;253:750-62.

146. Katz P, Zaytoun AM, Fauci AS. Mechanisms of human cell-mediated cytotoxicity I. Modulation of natural killer cell activity by cyclic nucleotides. J Immunol 1982;129:287-96.

147. Roder JC, Helfand SL, Werkmeister J, McGarry R, Beaumont TJ, Duwe A. Oxygen intermediates are triggered early in the cytolytic pathway of human NK cells. Nature 1982;298:569-72.

148. Suthanthiran M, Solomon SD, Wiliams PS, Rubin AL, Novogrodsky A, Stenzel KH. Hydroxyl radical scavengers inhibit human natural killer cell activity. Nature 1984;307:276-8.

149. Duwe AK, Werkmeister J, Roder JC, Lauzon R, Payne U. Natural killer cell-mediated lysis involves an hydroxyl radical-dependent step. J Immunol 1985;134:2637-44.

150. Fong KL, McCay PB, Poter JR, Keele BB, Misra H. Evidence that peroxidation of lysosomal membrane is initiated by hydroxyl free radicals produced during flavin enzyme activity. J Biol Chem 1973;248:7792-7.

151. Pohajdak B, Gomez JL, Wilkins JA, Greenberg AH. Tumor-activated NK cells trigger monocyte oxidative metabolism. J Immunol 1984;133:2430-6.

152. El-Hag A, Clark RA. Intact natural killer activity in chronic granulomatous disease: evidence against an oxygen-dependent cytotoxic mechanism. J Immunol 1984;132:569-70.

153. Metz SA. Anti-inflammatory agents as inhibitors of prostaglandin synthesis in man. Med Clin North Am 1981;65:713-58.

154. Carpen O, Virtanea I, Saksela E. Ultrastructure of human natural killer cells: nature of the cytolytic contacts in relation to cellular secretion. J Immunol 1982;128:2691-7.

155. Carpen O, Virtanen I, Saksela E. The cytotoxic activity of human natural killer cells requires an intact secretory apparatus. Cell Immunol 1981;58:97-106.

156. Verhoef J, Sharma SD. Inhibition of human natural killer activity by lysosomotrophic agents. J Immunol 1983;131:125-31.

157. Abo T, Roder JC, Abo W, Cooper M, Balch CM. Natural killer (HNK-1^+) cells in Chediak-Higashi patients are present in normal numbers but are abnormal in function and morphology. J Clin Invest 1982;70:193-7.

158. Roder J, Duwe A. The beige mutation in the mouse selectively impairs natural killer function. Nature 1979;278:451-3.

159. Young JD, Leong LG, Liu CC, Damiano A, Cohn ZA. Extracellular release of lymphocyte cytolytic pore-forming protein (perforin) after ionophore stimulation. Proc Nat Acad Sci USA 1986;83:5668-72.

160. Podack ER, Dennert G. Assembly of two types of tubules with putative cytolytic function by cloned NK cells. Nature 1983;302:442-5.

161. Bishop CJ, Whiting VA. The role of natural killer cells in the intravascular death of intravenously injected murine tumour cells. Brit J Cancer 1983;48:441-4.

162. Young JD, Cohn ZA. Cell-mediated killing: a common mechanism? Cell 1986:46:641-2.

163. Herberman RB, Reynolds CW, Ortaldo JR. Mechanism of cytotoxicity by natural killer (NK) cells. Ann Rev Immunol 1986;4:651-80.

164. MacDermott RP, Schmidt RE, Caulfield JP, Hein A, Bartley GJ, Ritz J, Schlossman SF, Austin KF, Stevens RL. Proteoglycans in cell-mediated cytotoxicity. Identification, localization, and exocytosis of a chondroitin sulfate proteoglycan from human cloned natural killer cells during target cell lysis. J Exp Med 1985;162:1771-87.

165. Farram E, Targan SR. Identification of human natural killer soluble cytotoxic factor(s) (NKCF) derived from NK-enriched lymphocyte populations: specifity of generation and killing. J Immunol 1983;130:1252-6.

166. Wright SC, Bonavida B. Studies on the mechanism of natural killer cell-mediated cytotoxicity. V. Lack of NK specificity at the level of induction of natural killer cytotoxic factors in cultures of human, murine, or rat effector cells stimulated with mycoplasma-free cell lines. J Immunol 1984;133:3415-23.

167. Werkmeister JA, Roder JC, Curry C, Pross H. The effect of unphosphorylated and phosphorylated sugar moieties on human and mouse natural killer cell activity: is there selective inhibition at the level of target recognition and lytic recognition site? Cell Immunol 1983;80:172-86.

168. Bonavida B, Wright SC. Role of natural killer cytotoxic factors in the mechanism of target-cell killing by natural killer cells. J Clin Immunol 1986;6:1-8.

169. Ortaldo JR, Ransom JR, Sayers TJ, Herberman RB. Analysis of cytostatic/cytotoxic lymphokines: relationship of natural killer cytotoxic factor to recombinant lymphotoxin, recombinant tumor necrosis factor, and leukoregulin. J Immunol 1986;137:2857-63.

170. Bonavida B, Katz J, Gottlieb M. Mechanism of defective NK cell activity in patients with acquired immunodeficiency syndrome (AIDS) and AIDS-related complex I. Defective trigger on NK cells for NKCF production by target cells, and partial restoration by IL 2. J Immunol 1986;137:1157-63.

171. Ransom JH, Evans CH, McCabe RP, Pomato N, Heinbaugh JA, Chin M, Hanna MG. Leukoregulin, a direct-acting anticancer immunological hormone that is distinct from lymphotoxin and interferon. Cancer Res 1985;45:851-62.

172. Le J, Vilcek J. Biology of disease. Tumour necrosis factor and interleukin 1: cytokines with multiple overlapping biological activities. Lab Invest 1987;56:234-48.

173. Peters PM, Ortaldo JR, Shalaby MF, Svedersky LP, Nedwin GE, Bringman TS, Hass PE, Aggarwal BB, Herberman RB, Goeddel DV, Palladino MA. Natural killer-sensitive targets stimulate production of TNF-α but not TNF-β (Lymphotoxin) by highly purified human peripheral blood large granular lymphocytes.
J Immunol 1986;137:2592-8.

174. Hudig DH, Redelman D, Minning LL. The requirement for proteinase activity for human lymphocyte-mediated natural cytotoxicity (NK): evidence that the proteinase is serine dependent and has aromatic amino acid specificity of cleavage. J Immunol 1984:133;2647-54.

175. Hudig D, Haverty T, Fulcher C, Redelman D, Mendensohn J. Inhibition of human natural cytotoxicity by macromolecular antiproteases.
J Immunol 1981;126:1569-74.

176. Zucker-Franklin D, Grusky G, Yang JS. Arylsulfatase in natural killer cells: Its possible role in cytotoxicity.
Proc Nat Acad Sci USA. 1983:80;6977-81.

177. Ullberg M, Jondall M. Recycling and target binding capacity of human natural killer cells. J Exp Med 1981;153:615-28

178. Targan S, Decker JM, Ades EW. Mechanism of inhibition of natural killing by a glycopeptide isolated from the K562 plasma membrane.
Nat Immun Cell Growth Regul 1983/84;3:113-23.

179. Hercend T, Schmidt R, Brennan A, Edson MA, Reinherz EL, Schlossman SF, Ritz J. Identification of a 140-kDa activation antigen as a target structure for a series of human cloned natural killer cell lines.
Eur J Immunol 1984;14:844-52.

180. Haller O, Hansson M, Kiessling R, Wigzell H. Role of non-conventional natural killer cells in resistance against syngeneic tumour cells in vivo. Nature 1977;270:609-11.

181. Talmadge JE, Meyers KM, Prieur DJ, Starkey JR. Role of NK cells in tumour growth and metastasis in beige mice. Nature 1980;284:622-4.

182. Karre K, Klein GO, Kiessling R, Klein G, Roder JC. Low natural in vivo resistance to syngeneic leukaemias in natural killer-deficient mice. Nature 1980;284:624-6.

183. Warner JF, Dennert G. Effects of a cloned cell line with NK activity on bone marrow transplants, tumour development and metastasis in vivo. Nature 1982;300:31-4.

184. Hanna N. In vivo activities of NK cells against primary and metastatic tumours in experimental animals. In: Lotzova E, Herberman RB (eds) Immunobiology of Natural Killer Cells, CRC Press Inc, Florida, 1986, Vol II, 1-10.

185. Hanna N, Schneider M. Enhancement of tumour metastasis and suppression of natural killer cell activity by β-estradiol treatment. J Immunol 1983;130:974-980.

186. Hanna N, Fidler IJ. Relationship between metastatic potential and resistance to natural killer cell-mediated cytotoxicity in three murine tumour systems. J Nat Cancer Inst 1981;66:1183-90

187. Hanna N, Fidler IJ. The role of natural killer cells in the destruction of circulating tumour emboli. J Nat Cancer Inst 1980;65:801-7.

188. Kiessling R, Hansson M, Gronberg A. Natural killer cells as regulators of malignant and normal cell growth. In: Yamamura Y, Tada T (eds) Progress In Immunology V, Academic Press, Tokyo, 1181-207.

189. Pross HF, Baines MG. Spontaneous human lymphocyte-mediated cytotoxicity against tumour target cells I. The effect of malignant disease. Int J Cancer 1976;18:593-604.

190. Saijo N, Shimizu E, Shikuya M, Irimajiri N, Takizawa T, Eguchi K, Shinkai T, Tominaga K, Shinabukuro Z, Taniguchi T, Hoshi A. The effect of chemotherapy on natural killer activity and antibody dependent cell-mediated cytotoxicity in carcinoma of the lung. Br J Cancer 1982;46:180-9.

191. Turoz T, Dokhelar MC, Lipinski M, Amiel JL. Low natural killer cell activity in patients with malignant lymphoma. Cancer 1985;50:2333-5.

192. Lotzova E, Savary CA, Herberman RB. Induction of NK cell activity against fresh human leukemia in culture with interleukin 2. J Immunol 1987;138:2718-27.

193. Steinhauer EH, Doyle AT, Reed J, Kadish AS. Defective natural cytotoxicity in patients with cancer: normal number of effector cells but decreased recycling capacity in patients with advanced disease. J Immunol 1982;129:2255-9.

194. Kadish AS, Doyle AT, Steinhauer EH, Ghossein NA. Natural cytotoxicity and interferon production in human cancer: deficient natural killer cell activity and normal interferon production in patients with advanced disease. J Immunol 1981;127:1817-22.

195. Balch CM, Tilden AB, Dougherty PA, Cloud GA, Abo T. Depressed levels of granular lymphocytes with natural killer (NK) cell-function in 247 cancer patients. Ann Surg 1983;178:192-9.

196. Hersey P, Edwards A, McCarthy W, Milton G. Tumor related changes and prognostic significance of natural killer cell activity in melanoma patients. In: Herbermen RB (ed) NK Cells and Other Natural Effector Cells, Academic Press, New York, 1982, 1167-74.

197. Hogan PG, Hapel AJ, Doe WF. Intestinal lymphokine-activated killer (LAK) cells-cytotoxicity for colon cancer cells and modulation of their generation (abstr). Gastroenterology 1986;90:1462.

198. Robinson BWS, Nicod LP, Lipscomb MF, Loews GB. Natural killer cells are present in the normal human lung but are functionally impotent. J Clin Invest 1984;74:942-50.

199. Vose BM, Moore M. Suppressor cell activity of lymphocytes infiltrating human lung and breast tumours. Int J Cancer 1979;24:579-585.

200. Vose BM, Moore M. Natural cytotoxicity in humans: susceptibility of freshly isolated tumor cells to lysis. J Nat Cancer Inst 1980;65:257-63.

201. Serrate SA, Vose BM, Timonen T, Ortaldo JR, Herberman RB. Association of human natural killer cell activity against primary tumors with large granular lymphocytes. In: Herbermen RB (ed) NK Cells and Other Natural Effector Cells, Academic Press, New York, 1982, 1055-60.

202. Vose BM, Moore M. Human tumor-infiltrating lymphocytes: a marker of host response. Sem Haematol 1985;22:27-40.

203. Vanky F, Klein E. Auto-tumor lytic potential of lymphocytes separated from human solid tumors. In: Herbermen RB (ed) NK Cells and Other Natural Effector Cells, Academic Press, New York, 1982, 1061-8.

204. Golub SH, Niitsuma M, Kawate N, Cochran AJ, Holmes EC. NK activity of tumor infiltrating and lymph node lymphocytes in human pulmonary tumours. In: Herbermen RB (ed) NK Cells and Other Natural Effector Cells, Academic Press, New York, 1982, 1113-8.

205. Vose BM, Moore M. Natural cytotoxic effectors in human tumours and tumour draining nodes. In: Herbermen RB (ed) NK Cells and Other Natural Effector Cells, Academic Press, New York, 1982, 1127-32.

206. Vose BM, Gallagher P, Moore M, Schofield PF. Specific and nonspecific lymphocyte cytotoxic function in colon cancer.
Br J Cancer 1981;44:846-55.

207. Strayer DR, Carter WA, Mayberry SD, Pequignot E, Brodsky I. Low natural cytotoxicity of peripheral blood mononuclear cells in individuals with high familial incidences of cancer.
Cancer Res 1984;44:370-4.

208. Ferson F, Edwards A, Lind A, Milton GW, Hersey P. Low natural killer cell activity and immunoglobulin levels associated with smoking in human subjects. Int J Cancer 1979;23:603-9.

209. Hersey P, Bradley M, Hasic E, Haran G, Edwards A, McCarthy WH. Immunological effects of solarium exposure in normal human subjects. Lancet 1983;1:545-8.

210. Roder JC, Haliotis T, Laing L, Kozbor D, Rubin P, Pross H, Boxer LA. White JG, Fauci AS. Mostowski H, Matheson DS. Further studies of natural killer cell function in Chediak-Higashi patients. Immunology 1982;46:555-60.

211. Peter HH, Friedrich W, Dopfer R, Muller W, Kortman C, Pichler WJ, Heinz F, Rieger CHL. NK cell function in severe combined immunodeficiency (SCID): evidence of a common T and NK cell defect in some but not all SCID patients. J Immunol 1983;131:2332-8.

212. Ernst PB, Befus AD, Bienenstock J. Leukocytes in the intestinal epithelium: an unusual immunological compartment. Immunol Today 1985;6:50-5.

213. Nencioni L, Villa L, Boraschi D, Berti B, Tagliabue A. Natural and antibody-dependent cell-mediated activity against Salmonella Typhimurium by peripheral and intestinal lymphoid cells in mice. J Immunol 1983;130:903-7.

214. Klimpel GR, Niesel DW, Klimpel KD. Natural cytotoxic effector cell activity against shigella flexneri-infected HeLa cells. J Immunol 1986;136:1081-6.

215. Blanchard DK, Stewart WE, Klein TW, Friedman H, Djeu JY. Cytolytic activity of human peripheral blood leucocytes against Legionella pneumophila-infected monocytes: characterization of the effector cell and augmentation by interleukin 2. J Immunol 1987;139:551-6.

216. Hidore MR, Murphy JW. Natural cellular resistance of beige mice against Cryptococcus neoformans. J Immunol 1986;137:3624-31.

217. Hatcher FM, Kuhn RE. Destruction of Trypanosoma cruzi by natural killer cells. Science 1982;218:295-6.

218. Biron CA, Turgiss LR, Welsh RM. Increase in NK cell number and turnover rate during acute viral infection. J Immunol 1983;131:1539-45.

219. Shellam GR, Grundy JE, Harnett GB, Allan JE. Natural resistance against Cytomegalovirus and other viruses in mice. In: Yamamura Y, Tada T (eds) Progress in Immunology V, Academic Press, Tokyo, 1983,1209-17.

220. Fitzgerald PA, Mendelsohn M, Lopez C. Human natural killer cells limit replication of Herpes simplex virus type 1 in vitro. J Immunol 1985;134:2666-72.

221. Bandyopadhyay S, Perussia B, Trinchieri G, Miller DS, Starr SE. Requirement for HLA-DR$^+$ accessory cells in natural killing of Cytomegalovirus-infected fibroblasts. J Exp Med 1986;164:180-95.

222. Lopez C, Kirkpatrick D, Fitzgerald P. The role of NK (HSV-1) effector cells in resistance to Herpesvirus infections in man. In: Herberman RB (ed) NK Cells and Other Natural Effector Cells, Academic Press, New York,1982,1445-50.

223. Shellam GR, Grundy JE, Allan JE. The role of natural killer cells and interferon in resistance to murine Cytomegalovirus. In: Herberman RB (ed) NK Cells and Other Natural Effector Cells, Academic Press, New York,1982,145-58.

224. Bukowski JF, Warner JF, Dennert G, Welsh RM. Adoptive transfer studies demonstrating the antiviral effect of natural killer cells in vivo. J Exp Med 1985;161:40-52.

225. Habu S, Akamatsu K, Tamaoki N, Okumura K. In vivo significance of NK cell on resistance against virus (HSV-1) infections in mice. J Immunol 1984;133:2743-7.

226. Bandyopadhyay S, Miller DS, Matsumoto-Kobayashi M, Clark SC, Starr SE. Effects of interferons and interleukin 2 on natural killing of cytomegalovirus-infected fibroblasts. Clin Exp Immunol 1987;67:372-82.

227. Welsh RM, Doe WF. Cytotoxic cells induced during lymphocytic choriomeningitis virus infection of mice : natural killer cell activity in cultured spleen leukocytes concomitant with T-cell-dependrent immune interferon production. Infect Immun 1980;30;473-83.

228. Bukowski JF, Woda BA, Habu S, Okumura K, Welsh RM. Natural killer cell depletion enhances virus synthesis and virus-induced hepatitis in vivo. J Immunol 1983;131:1531-8.

229. Lopez C, Fitzgerald PA, Siegal FP. Severe acquired immune deficiency syndrome in male homosexuals : diminished capacity to make interferon-alpha in vitro is associated with severe opportunistic infections. J Infect Dis 1983;148:961-6.

230. Fitzgerald PA, Lopez C. Natural killer cells active against viral, bacterial, protozoan, and fungal infections. In : Lotzova E, Herberman RB, eds. Immunobiology of Natural Killer Cells, CRC Press Inc., Florida, Vol II, 1986,107-31.

231. Bukowski JF, Welsh RM. Inability of interferon to protect virus-infected cells against lysis by natural killer (NK) cells correlates with NK cell-mediated antiviral effects in vivo. J Immunol 1985;135:3537-41.

232. Borysiewicz LK, Rodgers B, Morris S, Graham S, Sissons JG. Lysis of human cytomegalovirus infected fibroblasts by natural killer cells : demonstration of an interferon-independant component requiring expression of early viral proteins and characterization of effector cells. J Immunol 1985;134:2695-701.

233. Sissons JGP. The immunology of Cytomegalovirus infection. J Royal Coll Physicians 1986;20:40-4.

234. Santoli D, Trinchieri G, Lief FS. Cell-mediated cytotoxicity against virus infected target cells in humans I. Characterization of the effector lymphocyte. J Immunol 1978;121:526-31.

235. Yasukawa M, Zarling JM. Autologous herpes simplex virus-infected cells are lysed by human natural killer cells. J Immunol 1983;131:2011-6.

236. Santoli D, Trinchieri G, Koprowski H. Cell-mediated cytotoxicity against virus infected target cells in humans II. Interferon induction and activation of natural killer cells. J Immunol 1978;121:532-8.

237. Patel PC, Menzies J. Epstein-Barr virus (EBV)-lymphoid cell interactions. II The influence of the EBV replication cycle on natural killing and antibody-dependent celllular cytotoxicity against EBV-infected cells. Clin Exp Immunol 1982;48:589-601

238. Ruscetti FW, Mikovits JA, Kalyanaraman VS, Overton R, Stevenson H, Stromberg K, Herberman RB, Farrar WL, Ortaldo J. Analysis of effector mechanisms against HTLV-I and HTLV-III/LAV-infected cells. J Immunol 1986;136:3619-24.

239. Burlington DB, Djeu JY, Wells MA, Kiley SC, Quinnan GV. Large granular lymphocytes provide an accessory function in the in vitro development of influenza A virus-specific cytotoxic T cells. J Immunol 1984;132:3154-8.

240. Nunn ME, Herberman RB, Holden HT. Natural cell-mediated cytotoxicity in mice against nonlymphoid tumor cells and some normal cells. Int J Cancer 1977;20:381-7.

241. O'Brein T, Kendra J, Stephens H, Knight R, Barrett AJ. Recognition and regulation of progenitor marrow elements by NK cells in the mouse. Immunology 1983;49:717-25.

242. Ohno A, Amos DB, Koren HS. Selective cellular natural killing against human leukaemic T cells and thymus. Nature 1977;266:547-8.

243. Gordon MY, Hibbin JA, Kearney LU, Gordon-Smith EC, Goldman JM. Colony formation by primitive haemopoietic progenitors in cocultures of bone marrow cells and stromal cells. Brit J Haem 1985;60:129-36.

244. Metcalf D. The role of the colony-stimulating factors in resistance to acute infections. Immunol Cell Biol 1987;65:35-43.

245. Hansson M, Beran M, Andersson B, Kiessling R. Inhibition of in vitro granulopoiesis by autologous allogeneic human NK cells. J Immunol 1982;129:126-32.

246. Herrmann F, Schmidt RE, Ritz J, Griffin JD. In vitro regulation of human haematopoiesis by natural killer cells : analysis at a clonal level. Blood 1987;69:246-54.

247. Mangan KF, Hartnett ME, Matis SA, Winkelstein A, Abo T. Natural killer cells suppress human erythroid stem cell proliferation in vitro. Blood 1984;63:260-9.

248. Holmberg LA, Miller BA, Ault KA. The effect of natural killer cells on the development of syngeneic hematopoietic progenitors. J Immunol 1984;133:2933-9.

249. Degliantoni G, Perussia B, Mangoni L, Trinchieri G. Inhibition of bone marrow colony formation by human natural killer cells and by natural killer cell-derived colony-inhibiting activity. J Exp Med 1985;161:1152-68.

250. Riccardi C, Santoni A, Barlozzari T, Herberman RB. In vivo reactivity of mouse natural killer (NK) cells against normal bone marrow cells. Cell Immunol 1981;60:136-43.

251. O'Brein TK, Kendra JA, Stephens HAF, Knight RA, Barrett AJ: Recognition of marrow elements by natural killer cells : are NK cells involved in haemopoietic regulation ? Brit J Haem 1983;53:161-4.

252. Degliantoni G, Murphy M, Kobayashi M, Francis M, Perussia B, Trinchieri G. Natural killer (NK) cell-derived hematopoietic colony-inhibiting activity and NK cytotoxic factor. Relationship with tumor necrosis factor and synergism with immune interferon. J Exp Med 1985;162:1512-30.

253. Broxmeyer HE, Lu L, Platzer P, Feit C, Juliano L, Rubin BY. Comparative analysis of the influences of human gamma, alpha and beta interferons on human multipotential (CFU-GEMM), erythroid (BFU-E), and granulocyte-macrophage (CFU-GM) progenitor cells. J Immunol 1978;131:1300-5.

254. Pistoia V, Ghio R, Nocera A, Leprini A, Perata A, Ferrarini M. Large granular lymphocytes have a promoting activity on human peripheral blood erythroid burst forming units. Blood 1985;65:464-72.

255. Kasahara T, Djeu JY, Dougherty SF, Oppenheim JJ. Capacity of human large granular lymphocytes (LGL) to produce multiple lymphokines : interleukin 2, interferon, and colony stimulating factor. J Immunol 1983;131:2379-85.

256. Kiessling R, Hochman PS, Haller O, Shearer GM, Wigzell H, Cudkowicz G. Evidence for a similiar or common mechanism for natural killer cell activity and resistance to hemopoietic grafts. Eur J Immunol 1977;7:655-63.

257. Lotzova E, Savary CA, Pollack SB. Prevention of rejection of allogeneic bone marrow transplants by NK 1.1 antiserum. Transplantation 1983;35:490-4.

258. Lopez C, Kirkpatrick D, Sorell M, O'Reilly RJ, Ching C. Association between pre-transplant natural kill and graft-versus-host disease after stem-cell transplantation. Lancet 1979;24:1103-6.

259. Dokhelar MC, Wiels J, Lipinski M, Tetaud C, Devergie A, Gluckman E, Tursz T. Natural killer cell activity in human bone marrow recipients. Early reappearance of peripheral natural killer cell activity in graft-versus-host disease. Transplantation 1981;31:61-5.

260. Charley MR, Mikhael A, Bennett M, Gilliam JN, Sontheimer RD. Prevention of lethal, minor-determinate, graft-vs-host disease in mice by the in vivo administration of anti-asialo GM. J Immunol 1983;131:2101-3.

261. Brenner MK, Reittie JE, Grob JP, Wimperis JZ, Stephens S, Patterson J, Hoffbrand AV, Prentice HG. The contribution of large granular lymphocytes to B cell activation and differentiation after T-cell-depleted allogeneic bone marrow transplantation. Transplantation 1986;42:257-61.

262. Scala G, Allavena P, Ortaldo JR, Herberman RB, Oppenheim JJ. Subsets of human large granular lymphocytes (LGL) exhibit accessory cell functions. J Immunol 1985;134:3049-55.

263. Suzuki R, Suzuki S, Ebina N, Kumagai K. Suppression of alloimmune cytotoxic T lymphocyte (CTL) generation by depletion of NK cells and restoration by interferon and/or interleukin 2. J Immunol 1985;134:2139-48.

264. Arai S, Yamamoto H, Itah K, Kumagi K. Suppressive effect of human natural killer cells on pokeweed mitogen-induced B cell differentiation. J Immunol 1983;131:651-7.

265. Nabel G, Allard WJ, Cantor H. A cloned cell line mediating natural killer cell function inhibits immunoglobulin secretion. J Exp Med 1982;156:658-63.

266. Tilden AB, Abo T, Balch CM. Suppressor cell function of human granular lymphocytes identified by the HNK-1 (leu 7) monoclonal antibody. J Immunol 1983;130:1171-5.

267. Pope RM, McChesney L, Stebbing N, Goldstein L, Talal N. Regulation of T cell proliferation by cloned interferon-α mediated by Leu-11b positive cells. J Immunol 1985;135:4048-53.

268. Abruzzo LV, Rowley DA. Homeostasis of the antibody response : immunoregulation by NK cells. Science 1983;222:581-5.

269. Brieva JA, Targan S, Stevens RH. NK and T cell subsets regulate antibody production by human in vivo antigen-induced lymphoblastoid B cells. J Immunol 1984;132:611-5.

270. Tovar Z, Pope RM, Talal N. Modulation of spontaneous immunoglobulin production by natural killer cells in rheumatoid arthritis. Arthritis Rheum 1986;29:1435-9.

271. Shah PD, Gilbertson SM, Rowley DA. Dendritic cells that have interacted with antigen are targets for natural killer cells. J Exp Med 1985;162:625-36.

272. Targan S, Brieva J, Newman W, Stevens R. Is the NK lytic process involved in the mechanism of NK suppression of antibody-producing cells ? J Immunol 1985;134:666-9.

273. Djeu JY, Timonen T, Herberman RB. Production of interferon by human natural killer cells in response to mitogens, viruses and bacteria. In: Herberman RB, ed. NK Cells And Other Effector Cells. New York, Academic Press, 1982, 669-74.

274. Harfast B, Huddlestone JR, Casali P, Merigan TC, Oldstone MBA. Interferon acts directly on human B lymphocytes to modulate immunoglobulin synthesis. J Immunol 1981;127:2146-50.

275. Pistoia V, Cozzolino F, Torcia M, Castigli E, Ferrarini M. Production of B cell growth factor by a Leu-7$^+$, OKM1$^+$ non-T cell with the features of large granular lymphocytes (LGL). J Immunol 1985;143:3179-84.

276. Farrar JJ, Benjamin WR, Hilfiker ML, Howard M, Farrar WL, Fuller-Farrar J. The biochemistry, biology, and role of interleukin 2 in the induction of cytotoxic T cell and antibody-forming B cell responses. Immunol Rev. 1982;63:129-66.

277. Poppema S, Visser L, De Leij L. Reactivity of presumed anti-natural killer cell antibody Leu 7 with intrafollicular T lymphocytes. Clin Exp Immunol 1983;54:834-7.

278. Pizzolo G, Semenzato G, Chilosi M, Morittu L, Ambrosetti A, Warner N, Bofill M, Janossy G. Distibution and heterogeneity of cells detected by HNK-1 monoclonal antibody in blood and tissues in normal, reactive and neoplastic conditions. Clin Exp Immunol 1984;57:195-206.

279. Scala G, Allavena P, Djeu JY, Kasahara T, Ortaldo JR, Herberman RB, Oppenheim JJ. Human large granular lymphocytes (LGL) are potent producers of interleukin 1. Nature 1984;309:56-9.

280. Greenberg AH, Khalil N, Pohajdak B, Talgoy M, Henkart P, Orr W. NK-leukocyte chemotactic factor (NK-LCF) : a large granular lymphocyte (LGL) granule-associated chemotactic factor. J Immunol 1986;137:3224-30.

281. Goldfarb RH, Timonen T, Herberman RB. Production of plasminogen activator by natural killer cells Large granular lymphocytes. J Exp Med 1984;159:935-51.

282. Scala GS, Djeu JY, Allavena P, Kasahara T, Ortaldo JR, Herberman RB, Oppenheim JJ. Cytokine secretion and noncytotoxic functions of human large granular lymphocytes. In: Lotzova E, Herberman RB (eds) Immunobiology of Natural Killer Cells, CRC Press Inc., Florida, Vol II, 1986,89-105.

283. Munakata T, Semba U, Shibuya Y, Kuwano K, Akagi M, Arai S. Induction of interferon-γ production by human natural killer cells stimulated by hydrogen peroxide. J Immunol 1985;134:2449-55.

284. Herberman RB, Hiserodt J, Balch C, Lotzova E, Bolhuis R, Glub S, Lanier LL, Phillips JH, Riccardi C, Ritz, Santoni A, Schmidt RE, Uchida A. Lymphokine-activated killer cell activity. Characteristics of effector cells and their progenitors in blood and spleen. Immunol Today 1987;8:178-81.

285. Burns GF, Good MF, Riglar C, Bartlett PF, Crapper RM, Mackay IR. Activated lymphocyte killer cells derived from melanoma tissue or peripheral blood. Clin Exp Immunol 1984;57:487-94.

286. Hsieh KH, Shu S, Lee CS, Chu CT, Yang CS, Chang K. Lysis of primary hepatic tumours by lymphokine activated killer cells. Gut 1987;28:117-24.

287. Oshimi K, Oshimi Y, Akutsu M. Takei Y, Saito H, Okada M, Mizoguchi H. Cytotoxicity of interleukin 2-activated lymphocytes and lymphoma cells. Blood 1986;68:938-48.

288. Grimm EA, Wilson D. The human lymphokine-activated killer cell system V. Purified recombinant interleukin 2 activates cytotoxic lymphocytes which lyse both natural killer-resistant autologous and allogeneic tumours and trinitrophenyl-modified autologous peripheral blood lymphocytes. Cell Immunol 1985;94:568-78.

289. Sondel PM, Hank JA, Kohler PC, Chen BP, Minkoff DZ, Molenda JA. Destruction of autologous human lymphocytes by interleukin 2-activated cytotoxic cells. J Immunol 1986;137:502-11.

290. Kane KP, Clark WR. Class I MHC antigens in the generation and expression of promiscuous cytotoxic cell function. J Immunol 1986;137:3080-86.

291. Damle NK, Doyle LV, Bender JR, Bradley EC. Interleukin 2-activated human lymphocytes exhibit enhanced adhesion to normal vascular endothelial cells and cause their lysis. J Immunol 1987;138:1779-85.

292. Bolhuis RLH, Van de griend RJ, Ronteltap CPM. Clonal expansion of human B73.1-positive natural killer cells or large granular lymphocytes exerting strong antibody-dependent and independent cytotoxicity and occasionally lectin-dependent cytotoxicity. Nat Immun Growth Regul 1983/84;3:61-72.

293. Van de Griend RJ, Tax WJM, van Krimpen BA, Vreugdenhil RJ, Ronteltap CPM, Bolhuis R. Lysis of tumor cells by $CD3^{+}4^{-}8^{-}16^{+}$ T cell receptor $\alpha\beta^{-}$ clones, regulated via CD3 and CD16 activation sites, recombinant interleukin 2, and interferon β. J Immunol 1987;138:1627-33.

294. Teh HS, Teh SJ. Direct evidence for a two-signal mechanism of cytotoxic T lymphocyte activation. Nature 1980;285:163-5.

295. Grimm EA, Muul LM, Wilson DJ. The differential inhibitory effects exerted by cyclosporine and hydrocortisone on the activation of human cytotoxic lymphocytes by recombinant interleukin-2 versus allospecific CTL. Transplantation 1985;39:537-40.

296. Grimm EA, Robb RJ, Roth JA, Neckers LM, Lachman LB, Wilson DJ, Rosenberg SA. Lymphokine-activated killer cell phenomenon III. Evidence that IL-2 is sufficient for direct activation of peripheral blood lymphocytes into lymphokine-activated killer cells. J Exp Med 1983;158:1356-61.

297. Mule JJ, Smith CA, Rosenberg SA. Interleukin 4 (B cell stimulatory factor 1) can mediate the induction of lymphokine-activated killer cell activity directed against fresh tumor cells. J Exp Med 1987;166:792-7.

298. Burns GF, Werkmeister JA, Triglia T. High frequency of precursors of anomalous killer cells in human peripheral blood: evidence for T cell regulation. Cell Immunol 1984;89:202-11.

299. Sone S, Utsugi T, Nii A, Ogura T. Effects of human alveolar macrophages on the induction of lymphokine (IL-2)-activated killer cells. J Immunol 1987;139:29-34.

300. Mazumder A, Grimm EA, Rosenberg SA. Characterization of the lysis of fresh human solid tumours by autologous lymphocytes activated in vitro with phytohemagglutinin. J Immunol 1983;130:958-64.

301. Seeley J, Svedmyr E, Weiland O, Klein G, Moller E, Eriksson E, Andersson K, van der Waal L. Epstein Barr virus selective T cells in infectious mononucleosis are not restricted to HLA-A and B antigens. J Immunol 1981;127:293-300.

302. Strassman G, Bach FH, Zarling JM. Depletion of human NK cells with monoclonal antibodies allows the generation of cytotoxic T lymphocytes without NK-like cells in mixed cultures. J Immunol 1983;130:1556-60.

303. Mazumder A, Grimm EA, Rosenberg SA. Lysis of fresh solid tumor cells by autologous lymphocytes activated in vitro by allosensitization. Cancer Immunol Immunother 1983;15:1-10.

304. Burns GF, Triglia T, Werkmeister JA. In vitro generation of human activated lymphocyte killer cells: separate precursors and modes of generation of NK-like cells and "anomalous" killer cells. J Immunol 1984;133:1656-63.

305. Phillips JH, Le AM, Lanier LL. Natural killer cells activated in a human mixed lymphocyte response culture identified by expression of Leu-11 and class II histocompatibility antigens. J Exp Med 1984;159:993-1008.

306. Balsari A, Fossati G, Taramelli D, Tona G, Delia D, Giardini R, Parmiani G. Allostimulation of patients lymphocytes generates both T and NK-like cells cytotoxic for autologous melanoma. Br J Cancer 1985;52:73-80.

307. Moretta A, Pantaleo G, Mingari MC, Melioli G, Moretta L, Cerottini JC. Assignment of human natural killer (NK)-like cells to the T cell lineage. Single allospecific T cell clones lyse specific or NK-sensitive target cells via distinct recognition structures. Eur J Immunol 1984;14:121-5.

308. De Vries JE, Spits H. Cloned human cytotoxic T lymphocyte (CTL) lines reactive with autologous melanoma cells I. In vitro generation, isolation, and analysis to phenotype and specificity. J Immunol 1984;132:510-19.

309. Phillips JH, Lanier LL. Dissection of the lymphokine-activated killer phenomenon. Relative contribution of peripheral blood natural killer cells and T lymphocytes to cytolysis. J Exp Med 1986;164:814-825.

310. Brooks CG. Reversible induction of natural killer cell activity in cloned murine cytotoxic T lymphocytes. Nature 1983;305:155-8.

311. Tilden AB, Itoh K, Balch CM. Human lymphokine-activated killer (LAK) cells: identification of two types of effector cells. J Immunol 1987;138:1068-73.

312. Lanier LL, Federspiel NA, Ruitenberg JJ, Phillips JH, Allison JP, Littman D, Weiss A. The T cell antigen receptor complex expressed on normal peripheral blood $CD4^-$,$CD8^-$ T lymphocytes. A CD3-associated disulphide-linked g chain heterodimer. J Exp Med 1987;165:1076-94.

313. Ritz J, Campen TJ, Schmidt RE, Royer HD, Hercend T, hussey E, Reinherz EL. Analysis of T-cell receptor gene rearrangement and expression in human natural killer cell clones. Science 1985;228:1540-3.

314. Ferrini S, Miescher S, Zocchi MR, von Fliedner V, Moretta A. Phenotypic and functional characterization of recombinant interleukin 2 (rIL 2)-induced activated killer cells: analysis at the population and clonal levels. J Immunol 1987;138:1297-1302.

315. Haynes BF. Human T lymphocyte antigens as defined by monoclonal anyibodies. Immunol Rev 1981;57:127-161.

316. Zocchi MR, Bottino C, Ferrini S, Moretta L, Moretta A. A novel 120 kD surface antigen expressed by a subset of human lymphocytes. Evidence that lymphokine-activated killer cells express this molecule and use it in their effector function. J Exp Med 1987;166:319-26.

317. Grimm EA, Ramsey KM, Mazumder A, Wilson DJ, Djeu JY, Rosenberg SA. Lymphokine-activated killer cell phenomenon II. Precursor phenotype is serologically distinct from peripheral T lymphocytes, memory cytotoxic thymus-derived lymphocytes, and natural killer cells. J Exp Med 1983;157:884-897.

318. Damle NK, Doyle LV, Bradley EC. Interleukin 2-activated human killer cells are derived from phenotypically heterogeneous precursors. J Immunol 1986;137:2814-22.

319. Bersani L, Colotta F, Peri G, Mantovani A. Cytotoxic effector function of B lymphoblasts. J Immunol 1987;139:645-8.

320. Gray JD, Torten M, Golub SH. Generation of natural killer-like cytotoxicity from human thymocytes with Interleukin-2. Nat Immun Cell Growth Regul 1983/84;3:124-33.

321. Shanahan F, Brogan M, Targan S. Human mucosal cytotoxic effector cells. Gastroenterology 1987;92:1951-7.

322. Rosenberg SA, Spiess P, Lafreniere R. New approach to the adoptive immunotherapy of cancer with tumour-infiltrating lymphocytes. Science 1986;233:1318-21.

323. Miescher S, Whiteside TL, Moretta L, von Fliedner V. Clonal and frequency analysis of tumor-infiltrating T lymphocytes from human solid tumors. J Immunol 1987;138:4004-11.

324. Binz H, Fenner M, Frei D, Wigzell H. Two independent receptors allow selective target lysis by T cell clones. J Exp Med 1983;157:1252-60.

325. Werkmeister JA, Triglia T, Andrews P, Burns GF. Identification of a structure on human melanoma cells recognized by CTL exhibiting anomalous killer cell function. J Immunol 1985;135:689-95.

326. Hercend T, Meuer S, Brennan A, Edson MA, Acuto O, Reinherz EL, Schlossman SF, Ritz J. Identification of a clonally restricted 90kD heterodimer on two human cloned natural killer cell lines. Its role in cytotoxic effector function. J Exp Med 1983;158:1547-60.

327. Pawelec G, Newman W, Schwulera U, Wernet P. Heterogeneity of human natural killer recognition demonstrated by cloned effector cells and differential blocking of cytotoxicity with monoclonal antibodies. Cell Immunol 1985;92:31-40.

328. David V, Bourge JF, Guglielmi P, Mathieu-Mahul D, Degos L, Bensussan A. Human T cell clones use a CD3-associated surface antigen recognition structure to exhibit both NK-like and allogeneic cytotoxic reactivity. J Immunol 1987;138:2831-6.

329. Moingeon P, Nowill A, Courtois G, Azzarone B, Motte P, Ythier A, Bohuon C, Hercend T. A target structure for a series of human cloned natural killer cell lines is recognized by both anti TNKtar and 4F2 monoclonal antibodies. J Immunol 1985;134:2930-4.

330. Lanzavecchia A. Is the T-cell receptor involved in T-cell killing? Nature 1986;319:778-80.

331. Van De Griend RJ, Bolhuis RLH, Stoter G, Roozemond RC. Regulation of cytolytic activity in $CD3^-$ and $CD3^+$ killer cell clones by monoclonal antibodies (anti-CD16, anti-CD2, anti-CD3) depends on subclass specificty of target cell IgG-FcR. J Immunol 1987;138:3137-44.

332. Mule JJ, Shu S, Rosenberg SA. The antitumor efficacy of lymphokine-activated killer cells and recombinant interleukin 2 in vivo. J Immunol 1985;135:646-52.

333. Ettinghausen SE, Lipford EH, Mule J, Rosenberg SA. Recombinant interleukin 2 stimulates in vivo proliferation of adoptively transferred lymphokine-activated killer cells (LAK) cells. J Immunol 1985;135:3623-35.

334. Merrill JE. The implications of natural killer (NK) cell activity in nonmalignant chronic diseases. In: Lotzova E, Herberman RB (eds) Immunobiology of Natural Killer Cells, CRC Press Inc, Florida,1986, Vol II,75-87.

335. Tsokos GC, Balow JE. Cellular immune responses in SLE. Progress in Allergy 1984;35:93-161.

336. MacDermott RP, Bragdon MJ, Kodner IJ, Bertovich MJ. Deficient cell-mediated cytotoxicity and hyporesponsiveness to interferon and mitogenic lectin activation by inflammatory bowel disease peripheral blood and intestinal mononuclear cells. Gastroenterology 1986;90:6-11.

337. Vierling JM, Nelson DL, Strober W, Bundy BM, Jones EA. In vitro cell-mediated cytotoxicity in primary biliary cirrhosis and chronic hepatitis: dysfunction of spontaneous cell-mediated cytotoxicity in primary biliary cirrhosis. J Clin Invest 1977;60:1116-28.

338. Katz P, Simone CB, Henkart PA, Fauci AS. Mechanisms of antibody-dependent cellular cytotoxicity. The use of effector cells from chronic granulomatous disease patients as investigative probes. J Clin Invest 1980;65:55-63.

339. Ritz J, Schlossman SF. Utilization of monoclonal antibodies in the treatment of leukaemia and lymphoma. Blood 1982;59: 1-11.

340. Grillot-courvalin C, Vinci G, Tsapis A, Dokhelar MC, Vainchenker W, Brouet JC. The syndrome of T8 hyperlymphocytosis: variation in phenotype and cytotoxic activities of granular cells and evaluation of their role in associated neutropenia. Blood 1986;69:1204-10.

341. Rambaldi A, Pelicci PG, Allavena P, Knowles DM, Rossini S, Bassan R, Barbui T, Dalla-Favera R, Mantovani A. T cell receptor β chain gene rearrangements in lymphoproliferative disorders of large granular lymphocytes/natural killer cells. J Exp Med 1985;162: 2156-2162.

342. Tagawa S, Tokumine Y, Ueda E, Waki K, Kanayama Y, Taniguchi N, Nakanishi T, Inoue R, Kitani T. Leu 11^+ Tγ cell chronic lymphocytic leukaemia with partially activated natural killer function and its further activation by recombinant IL-2 in vitro. Blood 1986;68: 846-52.

343. Chang AE, Hyatt CL, Rosenberg SA. Systemic administration of recombinant human interleukin-2 in mice. J Biol Res Mod 1984;3:561-72.

344. Yanagisawa M, Kato M, Ikeno K, Kobayashi T, Miyagawa Y, Komiyama A, Akabane T. Defective generation of killer cells against spontaneously Epstein-Barr virus (EBV)-transformed autologous B cells in a fatal EBV infection. Clin Exp Immunol 1987;68:251-8.

345. Carl M, Dasch GA. Characterization of human cytotoxic lymphocytes directed against cells infected with typhus group rickettsiae: evidence for lymphokine-activation of effectors. J Immunol 1986;136:2654-61.

346. Klein JR, Kagnoff MF. Nonspecific recruitment of cytotoxic effector cells in the intestinal mucosa of antigen-primed mice. J Exp Med 1984;160:1931-6.

347. Goto M, Zvaifler NJ. Characterization of the natural killer-like lymphocytes in rheumatoid synovial fluid. J Immunol 1985;134:1483-86.

348. Hirata I, Berrebi G, Austin LL, Keren DF, Dobbins WO. Immunohistological characterization of intraepithelial and lamina propria lymphocytes in control ileum and colon and in inflammatory bowel disease. Dig Dis Sci 1986;31:593-603.

349. Ballardini G, Bianchi FB, Doniach D, Mirakion R, Pisi E, Bottazo GF. Abberant expression of HLA-DR antigens on bile duct epithelium in primary biliary cirrhosis: relevance to its pathogenesis. Lancet;ii:1009-13.

350. MacDermott RP, Franklin GO, Jenkins KM, Kodner IJ, Nash GS, Weinrieb IJ. Human Intestinal Mononuclear Cells I. Investigation of antibody-dependent, lectin-induced, and spontaneous cell-mediated cytotoxic capabilities. Gastroenterology 1980;78:47-56.

351. Chiba M, Bartnik W, ReMine SG, Thayer WR, Shorter RG. Human colonic intraepithelial and lamina propial lymphocytes: cytotoxicity in vitro and the potential effects of the isolation method on their functional properties. Gut 1981;22:177-86.

352. Bland PW, Britton DC, Richens ER, Pledger JV. Peripheral, mucosal, and tumour-infiltrating components of cellular immunity in cancer of the large bowel. Gut 1981;22:744-51.

353. Falchuk ZM, Barnhard E, Machado I. Human colonic mononuclear cells: studies of cytotoxic function. Gut 1981;22:290-4.

354. Fiocchi C, Tubbs RR, Youngman KR. Human intestinal mononuclear cells exhibit lymphokine-activated killer cell activity. Gastroenterology 1985;88:625-37.

355. Gibson PR, Dow EL, Selby WS, Strickland RG, Jewell DP. Natural killer cells and sponaneous cell-mediated cytotoxicity in the human intestine. Clin Exp Immunol 1984;156:438-44.

356. Gibson PR, Jewell DP. Local immune mechanisms in inflammatory bowel disease and colorectal cancer. Natural Killer cells and their activity. Gastroenterology 1986;90:12-19.

357. MacDermott RP, Bragdon MJ, Kodner IJ, Bertovich MJ. Deficient cell-mediated cytotoxicity and hyporesponsiveness to interferon and mitogenic lectin activation by inflammatory bowel disease peripheral blood and intestinal mononuclear cells. Gastroenterology 1986;90:6-11.

358. Gibson PR, Hermanomicz A, Verharr HJJ, Ferguson DJP, Lopez Bernal A, Jewell DP. Isolation of intestinal mononuclear cells: factors released which affect lymphocyte viability and function. Gut 1985;26:60-8.

359. Gibson PR, Hermanowicz A, Jewell DP. Factors affecting the spontaneous cell-mediated cytotoxicity of intestinal mucosal cells. Immunology 1984; 53:267-74.

360. Targan S, Britvan L, Kendal R, Vimadalal S, Soll A. Isolation of spontaneous and interferon inducible natural killer like cells from human colonic mucosa: lysis of lymphoid and autologous epithelial target cells. Clin Exp Immunol 1983; 54:14-22.

361. Shorter RG, McGill DB, Bahn RC. Cytotoxicity of mononuclear cells for autologous colonic epithelial cells in colonic diseases. Gastroenterology 1984; 86:13-22.

362. Gibson PR, van de Pol E, Doe WF. Sensitivity of human colonic epithelial cells (CEC) to lysis by mononuclear cells (MNC) from peripheral blood (abstr). Gastroenterology 1987;92:1403.

363. Clancy R, Pucci A. Absence of K cells in human gut mucosa. Gut 1978;19:273-6.

364. Bookman MA, Bull DM. Characteristics of isolated intestinal mucosal lymphoid cells in inflammatory bowel disease. Gastroenterology 1979;77:503-10.

365. Fiocchi C, Battisto JR, Farmer RG. Gut mucosal lymphocytes in inflammatory bowel disease. Isolation and preliminary functional characterization. Dig Dis Sci 1979;9:705-17.

366. Chiba M, Shorter RG, Thayer WR, Bartnik W, ReMine S. K-cell activity in lamina propial lymphocytes from the human colon. Dig Dis Sci 1979;24:817-22.

367. Lanier LL, Phillips JH. Evidence for three types of human cytotoxic lymphocyte. Immunology Today 1986;7:132-4.

368. Phillips JH, Lanier LL. Lectin dependent and anti-CD3 induced cytotoxicity are preferentially mediated by peripheral blood cytotoxic T lymphocytes expressing Leu-7 antigen. J Immunol 1985; 136:1579-85.

369. James SP, Fiocchi C, Graeff AS, Strober W. Phenotypic analysis of lamina propria lymphocytes. Predominance of helper-inducer and cytolytic T-cell phenotypes and deficiency of suppressor-inducer phenotypes in Crohn's disease and control patients. Gastroenterology 1986;91:1483-9.

370. MacDermott RP, Bragdon MJ, Jenkins KM, Franklin GO, Shedlofsky S, Kodner IJ. Human intestinal mononuclear cells II. Demonstration of a naturally occurring subclass of T cells which respond in the allogeneic mixed leucocyte reaction but do not effect cell mediated lympholysis. Gastroenterology 1981;80:748-57.

371. Roche JK, Fiocchi C, Youngman K. Sensitization to epithelial antigens in chronic mucosal inflammatory disease. Characterization of human intestinal mucosa-derived mononuclear cells reactive with purified epithelial cell-associated components in vitro. J Clin Invest 1985;75:522-30.

372. Greenwood JH, Austin LL, Dobbins WO. In vitro characterization of human intestinal intraepithelial lymphocytes. Gastroenterology 1983;85:1023-35.

373. Cerf-Bensussan N, Guy-Grand D, Griscelli C. Intraepithelial lymphocytes of human gut: isolation, characterization and study of natural killer activity. Gut 1985;26:81-8.

374. Klein JR. Ontogeny of the Thy-1$^-$, Lyt-2$^+$ murine intestinal intraepithelial lymphocyte. Characterization of a unique population of thymus-independent cytotoxic effector cells in the intestinal mucosa. J Exp Med 1986;164:309-14.

375. Tagliabue A, Befus AD, Clark DA, Bienenstock J. Characteristics of natural killer cells in the murine intestinal epithelium and lamina propria. J Exp Med 1982; 155:1785-96.

376. Alberti S, Colotta F, Speafico F, Delia D, Pasqualetto E, Luini W. Large granular lymphocytes from murine blood and intestinal epithelium: comparison of surface antigens, natural killer activity and morphology. Clin Immunol Immunopathol 1985;36:227-38.

377. Tagliabue A, Luini D, Soldateschi D, Boraschi D. Natural killer activity of gut mucosal lymphoid cells in mice. Eur J Immunol 1981;11:919-22.

378. Parrot DMV, Tait C, Mackenzie S, Mount AM, Davies MDJ, Micklem HS. Analysis of the effector functions of different populations of mucosal lymphocytes. Ann N Y Acad Sci 1983;409:307-19.

379. Nauss KM, Pavlina TM, Kumar V, Newberne PM. Functional characteristics of lymphocytes isolated from the rat large intestine. Gastroenterology 1984;86:468-75.

380. Flexman JP, Shellam GR, Mayrhofer G. Natural cytotoxicity, responsiveness to interferon and morphology of intra-epithelial lymphocytes from the small intestine of the rat. Immunology 1983;48:733-4.

381. Klein JR, Lefrancois L, Kagnoff MJ. A murine cytotoxic T lymphocyte clone from the intestinal mucosa that is antigen specific for proliferation and displays broadly reactive inducible cytotoxic activity. J Immunol 1985;135:3697-703.

382. Tagliabue A, Villa L, Scapigliati G, Boraschi D. Peyer's patch lymphocytes express natural cytotoxicity but not natural killer activity. Nat Immun Cell Growth Regul 1983/84;3:95-101.

383. Djeu JY, Lanza E, Pastore S, Hapel AJ. Selective growth of natural cytotoxic but not natural killer effector cells in interleukin-3. Nature 1983; 306:788-91.

384. Collins JL, Kao MS, Patek PQ. Humans express natural cytotoxic cell activity that is similiar to murine natural cytotoxic cell activity. J Immunol 1987;138:4180-4.

385. Golder JP, Doe WF. Isolation and preliminary characterization of human intestinal macrophages. Gastroenterology 1983;84:795-802.

386. Stutman O. Current evidence for immunological surveillance against tumors in mice and possible role of NK and NC cells. In: Yamamura Y, Tada T (eds), Progress in Immunology V, Academic Press Inc., Tokyo, 1983,1195-1207.

387. Cerf-Bensussan N, Schneeberger EE, Bhan AK. Immunohistologic and immunoelectron microscopic characterization of the mucosal lymphocytes of the human small intestine by the use of monoclonal antibodies. J Immunol 1983;130:2615-22.

388. Fiocchi C, Youngman KR, Farmer RG. Immunoregulatory function of human intestinal mucosa lymphoid cells: evidence for enhanced suppressor cell activity in inflammatory bowel disease. Gut 1983;24:692-701.

389. Strickland RG, Jewell DP. Immunoregulatory mechanisms in nonspecific inflammatory bowel disease. Ann Rev Med 1983;34:195-204.

390. Gibson PR. Local immune mechanisms. In: Chapman RG, Jewell DP (eds) Topics in Gastroenterology II, Blackwell Scientific Publications, Oxford, 1983, 307-21.

391. Hume DA, Allan W, Hogan PG, Doe WF. Immunohistochemical characteristics of macrophages in human liver and gastrointestinal tract: expression of CD4, HLA-DR, OKM1 and the mature macrophage marker 25F9 in normal and diseased tissue. J Leucocyte Biol 1987;42:474-84.

392. McLean IW, Nakane PK. Periodate-lysine-paraformaldehyde fixative. A new fixative for immunoelectron microscopy. J Histochem Cytochem 1974;22:1077-83.

393. Zwaldo G, Brocker EV, von Bassewitz DB, Feige U, Sorg C. A monoclonal antibody to a differentiation antigen present on mature human macrophages and absent from monocytes. J Immunol 1985;134:1487-92.

394. Abo T, Balch CM. A differentiation antigen of human NK and K cells identified by a monoclonal antibody (HNK-1). J Immunol 1981;127:1024-9.

395. Selby WS, Janossy G, Bofill M, Jewell DP. Intestinal lymphocyte subpopulations in inflammatory bowel disease: an analysis by immunohistological and cell isolation techniques. Gut 1984;25:32-40.

396. Velardi A, Grossi CE, Cooper MD. A large subpopulation of lymphocytes with T helper phenotype (Leu3/T4$^+$) exhibits the property of binding to NK cell targets and granular lymphocyte morphology. J Immunol 1985;134:58-64.

397. Velardi A, Prchal JT, Prasthofer EF, Grossi CE. Expression of NK-lineage markers on peripheral blood lymphocytes with T-helper (Leu3$^+$/T4$^+$) phenotype in B cell chronic lymphocytic leukaemia. Blood 1985;65:149-55.

398. Velardi A, Mingari MC, Moretta L, Grossi C. Functional analysis of cloned germinal center CD4$^+$ cells with natural killer cell-related features. Divergence from typical T helper cells. J Immunol 1986;137:2808-13.

399. Ritchie AWS, James K, Micklem HS. The distribution and possible significance of cells identified in human lymphoid tissue by the monoclonal antibody HNK-1. Clin Exp Immunol 1983;51:439-47.

400. Elson CO, Kagnoff MF, Fiocchi C, Befus AD, Targan S. Intestinal immunity and inflammation: recent progress. Gastroenterology 1986;91:746-68.

401. Wood GS, Turner RR, Shiurba RA, Eng L, Warnke RA. Human dendritic cells and macrophages. In situ immunophenotypic definition of subsets that exhibit specific morphologic and microenviromental characteristics. Amer J Path 1985;119:73-82.

402. Masucci MG, Klein E, Argov S. Disappearance of the NK effect after explanation of lymphocytes and generation of similiar nonspecific cytotoxicity correlated to the level of blastogenesis in activated cultures. J Immunol 1980;124:2458-63.

403. Abo T, Balch C. Characterization of HNK-1 (Leu 7)$^+$ human lymphocytes. II. Distinguishing phenotypic and functional properties of natural killer cells from activated NK-like cells. J Immunol 1982;129:1758-61.

404. Strassman G, Bach FH, Zarling JM. Depletion of human NK cells with monoclonal antibodies allows the generation of cytotoxic T lymphocytes without NK-like cells in mixed cultures. J Immunol 1983;130:1556-60.

405. Yam LT, Li CY, Crosby WH. Cytochemical identification of monocytes and granulocytes. Am J Clin Pathol 1971;55:283-90.

400. Elson CO, Kagnoff MF, Fiocchi C, Befus AD, Targan S. Intestinal immunity and inflammation: recent progress. Gastroenterology 1986;91:746-68.

401. Wood GS, Turner RR, Shiurba RA, Eng L, Warnke RA. Human dendritic cells and macrophages. In situ immunophenotypic definition of subsets that exhibit specific morphologic and microenviromental characteristics. Amer J Path 1985;119:73-82.

402. Masucci MG, Klein E, Argov S. Disappearance of the NK effect after explanation of lymphocytes and generation of similiar nonspecific cytotoxicity correlated to the level of blastogenesis in activated cultures. J Immunol 1980;124:2458-63.

403. Abo T, Balch C. Characterization of HNK-1 (Leu 7)$^+$ human lymphocytes. II. Distinguishing phenotypic and functional properties of natural killer cells from activated NK-like cells. J Immunol 1982;129:1758-61.

404. Strassman G, Bach FH, Zarling JM. Depletion of human NK cells with monoclonal antibodies allows the generation of cytotoxic T lymphocytes without NK-like cells in mixed cultures. J Immunol 1983;130:1556-60.

405. Yam LT, Li CY, Crosby WH. Cytochemical identification of monocytes and granulocytes. Am J Clin Pathol 1971;55:283-90.

406. Ornstein L, Ansley H, Saunders A. Improving manual differential white cell counts with cytochemistry. Blood Cells 1976;2:557.

407. Lozzio CB, Lozzio BB. Cytotoxicity of a factor isolated from human spleen. J Nat Cancer Inst 1973;50:535-8.

408. Rabin H, Hopkins RF, Ruscetti FW, Neubauer RH, Brown RL, Kawakami TG. Spontaneous release of a factor with properties of T cell growth factor from a continuous line of primate tumour T cells. J Immunol 1981;127:1852-6.

409. Leonard WJ, Depper JM, Uchiyama T, Smith KA, Waldmann TA, Greene WC. A monoclonal antibody that appears to recognize the receptor for human T-cell growth factor; partial characterization of the receptor. Nature 1982;300:267-9.

410. Reinherz EL, Kung PC, Goldstein G, Schlossman SF. Separation of functional subsets of human T cells by a monoclonal antibody. Proc Nat Acad Sci USA. 1979;76:4061-5.

411. Zarling JM, Clouse KA, Biddison WE, Kung PC. Phenotypes of human natural killer cell populations detected with monoclonal antibodies. J Immunol 1981;127:2575-80.

412. Van Wauae JP, De Mey JR, Goossens JG. OKT3: A monoclonal anti-human T lymphocyte antibody with potent mitogenic properties. J Immunol 1980;124:2708-13.

413. Hapel AJ, Warren H, Golder JP, Doe WF. Depression of interleukin 2 (IL-2) production by lamina propria lymphocytes (LPL) in invasive colon cancer (abstr). Gastroenterology 1983;84:1182.

414. Fiocchi C, Hilfiker ML, Youngman KR, Doerder NC, Finke JH. Interleukin 2 activity of human intestinal mucosa mononuclear cells. Decreased levels in inflammatory bowel disease. Gastroenterology 1984;86:734-42.

415. Bartnik W, Remine SG, Chiba M, Thayer WR, Shorter RG. Isolation and characterization of colonic intraepithelial and lamina proprial lymphocytes. Gastroenterology 1980;78:976-85.

416. Selby WS, Janossy G, Goldstein G, Jewell DP. T lymphocyte subsets in human intestinal mucosa-the distribution and relationship to MHC-derived antigens. Clin Exp Immunol 1981;44:453-8.

417. Seaman WE, Gindhart TD, Blackman MA, Dalal B, Talal N, Werb Z. Suppression of natural killing in vitro by monocytes and polymorphonuclear leukocytes. J Clin Invest 1982;69:876-88.

418. Hammerstrom J. Human monocyte-mediated cytotoxicity to K-562 cells; activation by lymphokines. Scand J Immunol 1979;10:575-84.

419. Saksela E, Timonen T. Morphology and surface properties of human NK cells. In; Herberman RB (ed) Natural Cell-Mediated Immunity Against Tumours, Academic Press, New York, 1980,178-82.

420. Claman HN. Corticosteroids and lymphoid cells. N Eng J Med 1972;287:388-97.

421. Britton S, Palacios R. Cyclosporin A - Usefulness, risks and mechanism of action. Immunol Rev 1982;65:5-22.

422. Gibson PR, van de Pol E, Pullman W, Doe WF. Lysis of colonic epithelial cells by allogeneic mononuclear and lymphokine-activated killer cells derived from peripheral blood and intestinal mucosa - evidence against a pathogenic role in inflammatory bowel disease. (submitted).

423. Mazumder A, Rosenberg AS. Successful immunotherapy of natural killer-resistant established pulmanary melanoma metastases by the intravenous adoptive transfer of syngeneic lymphocytes activated in vitro by interleukin 2. J Exp Med 1984;159:495-507.

424. Pulman WE, Hapel AJ, Doe WF. Growth factor production by human lamina propria cells (LPC) (abstr). Gastroenterology 1987;92:1583.

425. Muul LM, Gately MK. Hydrocortisone suppresses the generation of nonspecific "anomalous" killers but not specific cytolytic T lymphocytes in human mixed lymphocyte-tumor cultures. J Immunol 1984;132:1202-7.

426. Palacios R, Sugawara I. Hydrocortisone abrogates proliferation of T cells in autologous mixed lymphocyte reaction by rendering the interleukin-2 producer T cells unresponsive to interleukin-1 and unable to synthesize the T cell growth factor. Scand J Immunol 1982;15:25-31.

427. Balow JE, Hunninghake GW, Fauci AS. Corticosteroids in human lymphocyte-mediated cytotoxic reactions. Transplantation 1977;23:322-8.

428. Warren HS, Pembrey RG. Cyclosporine inhibits a two signal mechanism for the generation of cytotoxic NK-like cells, from small lymphocyte precursors. Immunol Letters 1986;12:69-75.

429. Andrus L, Lafferty KJ. Inhibition of T-cell activity by cyclosporin A. Scand J Immunol 1982;15:449-58.

430. James SP, Fiocchi C, Graeff AS, Strober W. Immunoregulatory function of lamina propria T cells in Crohn's disease. Gastroenterology 1985;88:1143-50.

431. Elson CO, Machelski E, Weiserbs DB. T cell-B cell regulation in the intestinal lamina propria in Crohn's disease. Gastroenterology 1985;89:321-7.

432. Stobo JD, Tomasi TB, Huizenga KA, Spencer RJ, Shorter RG. In vitro studies of inflammatory bowel disease. Surface receptors of the mononuclear cell required to lyse allogeneic colonic epithelial cells. Gastroenterology 1976;70:171-6.

433. Kemler BJ, Alpert E. Inflammatory bowel disease: study of cell mediated cytotoxicity for isolated human colonic epithelial cells. Gut 1980;21:353-9.

434. James SP, Strober W. Cytotoxic lymphocytes and intestinal disease (Editorial). Gastroenterology 1986;90:235-40.

435. Pullman WE, Hapel AJ, Doe WF. Growth factor production by human lamina propria cells (abstr). Gastroenterology 1987;92:1583.

436. Pedro AT, Tetteroo C, Van Der Schoot E, Visser FJ, Bos MJ, Von Dem Borne AE. Three different types of Fcγ receptors on human leucocytes defined by workshop antibodies; $Fc\gamma R_{low}$ of neutrophils, $Fc\gamma R_{low}$ of K/NK lymphocytes, and FcγRII. In: McMichael AJ (ed) Leucocyte Typing III White Cell Differentiation Antigens, Oxford University Press, Oxford, 1987,702-6.

437. Van de Griend RJ, Van Krimpen BA, Ronteltap CPM, Bolhuis RLH. Rapidly expanded activated human natural killer cell clones have strong antitumor cell activity and have the surface phenotype of either T, non-T, or null cells. J Immunol 1984;132:3185-91.

438. Koren HS, Williams MS. Natural killing and antibody-dependent cellular cytotoxicity are mediated by different mechanisms and by different cells. J Immunol 1978;121:1956-60.

439. Klein E, Ben-Bassat H, Neumann H, Ralph P, Zeuthen J, Polliack A, Vanky F. Properties of the K562 cell line, derived from a patient with chronic myeloid leukaemia. Int J Cancer 1976;18:421-31.

440. Anderson CL. Isolation of the receptor for IgG from a human monocyte cell line (U937) and from human peripheral blood monocytes. J Exp Med 1982;156:1794-1805.

441. Looney RJ, Abraham GN, Anderson CL. Human monocytes and U937 cells bear two distinct Fc receptors for IgG. J Immunol 1986;136:1641-7.

442. Bagchi S, Das KM. Detection and partial characterization of Crohn's disease tissue specific proteins recognized by Crohn's disease sera. Clin Exp Immunol 1984;55:41-8.

443. Smith KA. Interleukin 2. Ann Rev Immunol 1984;2:319-33.

444. Meuer SC, Hussey RE, Penta AC, Fitzgerald KA, Stadler BM, Schlossman SF, Reinherz EL. Cellular origin of interleukin 2 (IL-2) in man : evidence for stimulus-restricted IL 2 production by $T4^+$ and $T8^+$ T lymphocytes. J Immunol 1982;129:1076-9.

445. Luger TA, Smolen JS, Chused TM, Steinberg AD, Oppenheim JJ. Lymphocytes with either the helper ($T4^+$) or suppressor ($T8^+$) phenotype produce interleukin 2 in culture. J Clin Invest 1982;70:470-3.

446. Morgan DA, Ruscetti FW, Gallo R. Selective in vitro growth of T lymphocytes from normal human bone marrows. Science 1976;193:1007-8.

447. Baker PE, Gillis S, Ferm MM, Smith KA. The effect of T cell growth factor on the generation of cytolytic T cells. J Immunol 1978;121:2168-73.

448. Ting C, Yang SS, Hargrove ME. Induction of suppressor T cells by interleukin 2. J Immunol 1984;133:261-6.

449. Mingari MC, Gerosa F, Carra G, Accolla RS, Moretta A, Zubler RH, Waldmann TA, Moretta L. Human interleukin-2 promotes proliferation of activated B cells via surface receptors similar to those of activated T cells. Nature 1984;312:641-3.

450. Nakagawa T, Nakagawa N, Goldstein H, Volkman DJ, Fauci AS. Demonstration that human B cells respond differently to interleukin 2 and B cell differentiation factor based on their stages of maturation. J Immunol 1986;137:3175-82.

451. London L, Perussia B, Trinchieri G. Induction of proliferation in vitro of resting human natural killer cells : IL 2 induces into cell cycle most peripheral blood NK cells, but only a minor subset of low density T cells. J Immunol 1986;137:3845-54.

452. Trinchieri G, Perussia B. Biology of disease. Human natural killer cells: biologic and pathologic aspects. Lab Invest 1984;50:489-513.

453. Holter W, Goldman CK, Casabo L, Nelson D, Greene WC, Waldmann TA. Expression of functional IL 2 receptors by lipopolysaccharide and interferon-γ stimulated human monocytes. J Immunol 1987;138:2917-22.

454. Kasahara T, Hooks JJ, Dougherty SF, Oppenheim JJ. Interleukin 2-mediated immune interferon (IFN- γ) production by human T cells and T cell subsets. J Immunol 1983;130:1784-9.

455. Nedwin GE, Svedersky LP, Bringman TS, Palladino MA, Goeddel DV. Effect of interleukin 2, interferon-γ, and mitogens on the production of tumor necrosis factors α and ß. J Immunol 1985;135:2492-7.

456. Flomenberg N, Welte K, Mertelsmann R, Kernan N, Ciobanu N, Venuta S, Feldman S, Kruger G, Kirkpatrick D, Dupont B, O'Reilly R. Immunologic effects of interleukin 2 in primary immunodeficiency diseases. J Immunol 1983;130:2644-50.

457. Linker-Israel M, Bakke AC, Rodanthi C, Kitridou RC, Gendler S, Gillis S, Horwitz DA. Defective production of interleukin 1 and interleukin 2 in patients with systemic lupus erythematosus (SLE). J Immunol 1983;130:2651-5.

458. Huang Y, Miescher PA, Zubler RH. The interleukin 2 secretion defect in vitro in systemic lupus erythematosus is reversible in rested cultured T cells. J Immunol 1986;137:3515-20.

459. Nakayama E, Asano S, Takuwa N, Yokota J, Miwa S. Decreased TCGF activity in the culture medium of PHA stimulated peripheral mononuclear cells from patients with metastatic cancer. Clin Exp Immunol 1983;51:511-6.

460. Auer IO, Roder A, Frohlich J. Immune status in Crohn's disease. VI. Immunoregulation evaluated by multiple, distinct T-suppressor cell assays of lymphocyte proliferation, and by enumeration of immunoregulatory T-lymphocyte subsets. Gastroenterology 1984;86:1531-43. 1983;130:1970-73.

461. Winawer SJ, Sherlock P. Malignant neoplasms of the small and large intestine. In : Sleisenger MH, Fordtran JS (eds), Gastrointestinal Disease, WB Saunders Co., Philadelphia,1983,1224-6.

462. Gillis S, Ferm MM, Ou W, Smith KA. T cell growth factor : parameters of production and a quantitative microassay for activity. J Immunol 1978;120:2027-32.

463. Clancy R, Cripps A, Chipchase H. Regulation of human gut B lymphocytes by T lymphocytes. Gut 1984;25:47-51.

464. Allavena P, Scala G, Djeu JY, Procopio AD, Oppenheim JJ, Herberman RB, Ortaldo JR. Production of multiple cytokines by clones of human large granular lymphocytes. Cancer Immunol Immunother 1985;19:121-26.

465. Werkmeister J, Zaunders J, McCarthy W, Hersey P. Characterization of an inhibitor of cell division released in tumour cell cultures. Clin Exp Immunol 1980;41:487-96.

466. Ebert EC, Roberts AI, O'Connel SM, Robertson FM, Nagase H. Characterization of an immunosuppressive factor derived from colon cancer cells. J Immunol 1987;138:2161-8.

467. Miescher S, Whiteside TL, Carrel S, von Fliedner V. Functional properties of tumor-infiltrating and blood lymphocytes in patients with solid tumors: effects of tumor cells and their supernatants on proliferative responses of lymphocytes. J Immunol 1986;136:1899-1907.

468. Hersey P, Bindon C, Czerniecki M, Spurling A, Wass J, McCarthy WH. Inhibition of Interleukin 2 production by factors released from tumor cells. J Immunol 1983;131: 2837-42.

469. Husby G, Strickland RG, Rigler GL, Peake GT, Williams RC. Direct immunochemical detection of prostaglandin-E and cyclic nucleotides in human malignant tumors. Cancer 1977;40:1629-42.

470. Chouaib S, Chatenoud L, Klatzmann D, Fradelizi D. The mechanisms of inhibition of human IL 2 production II. PGE_2 induction of suppressor T lymphocytes. J Immunol 1984;132:1851-7.

471. Paul WE, Ohara J. B-cell stimulatory factor-1/interleukin 4. Ann Rev Immunol 1987;5:429-59.

472. Chouaib S, Fdadelizi D. The mechanism of inhibition of human IL 2 production. J Immunol 1982;129:2463-8.

473. Alvarez JM, Silva A, De Landazuri MO. Human T cell growth factor I. Optimal conditions for its production. J Immunol 1979;123:977-83.

474. Bonnard GD, Yasaka K, Jacobson D. Ligand-activated T cell growth factor-induced proliferation : absorption of T cell growth factor by activated T cells. J Immunol 1979;123:2704-8.

475. Bradley TR, Metcalf D. The growth of mouse bone marrow cells in vitro. Aust J Exp Biol Med Sci 1966;44:287-300.

476. Nagata S, Tsuchiya M, Asano S, Kaziro Y, Yamazaki T, Yamamoto O, Hirata Y, Kubota N, Oheda M, Nomura H, Ono M. Molecular cloning and expression of cDNA for human granulocyte colony-stimulating factor. Nature 1986;319:415-8.

477. Kawasaki ES, Ladner MB, Wang AM, Van Arsdell J, Warren MK, Coyne MY, Schweickart VL, Lee M, Wilson KJ, Boosman A, Stanley ER, Ralph P, Mark DF. Molecular cloning of a complementary DNA encoding human macrophage-specific colony-stimulating factor (CSF-1). Science 1985;230:291-6.

478. Wong GG, Witek JS, Temple PA, Wilkens KM, Leary AC, Luxenberg DP, Jones SS, Brown EL, Kay RM, Orr EC, Shoemaker C, Golde DW, Kaufman RJ, Hewick RM, Clark SC, Wang EA. Human GM-CSF. Molecular cloning of the cDNA and purification of the natural and recombinant proteins. Science 1985;228:810-5.

479. Dorssers L, Burger H, Bot F, Delwel R, Geurts van Kessel A, Lowenberg B, Wagemaker G. Characterization of a human multi-CSF cDNA clone identified by a conserved noncoding sequence in mouse interleukin 3. Gene 1987;55:115-24.

480. Metcalf D. The molecular biology and functions of the granulocyte-macrophage colony-stimulating factors. Blood 1986;67:257-67.

481. Metcalf D. The role of the colony-stimulating factors in resistance to acute infections. Immunol Cell Biol 1987;65:35-43.

482. Metcalf D, Begley CG, Johnson GR, Nicola NA, Vadas MA, Lopez AF, Williamson DJ, Wong GG, Clark SC, Wang EA. Biologic properties in vitro of a recombinant human granulocyte-macrophage colony-stimulating factor. Blood 1986;67:37-45.

483. Silberstein DS, Owen WF, Gasson JC, Dipersio JF, Golde DW, Bina JC, Soberman R, Austen KF, David JR. Enhancement of human eosinophil cytotoxicity and leukotriene synthesis by biosynthetic (recombinant) granulocyte-macrophage colony-stimulating factor. J Immunol 1986;137:3290-4.

484. Lopez AF, Williamson DJ, Gamble JR, Begley CG, Harian JM, Klebanoff SJ, Waltersdorph A, Wong G, Clark SC, Vadas M. Recombinant human granulocyte-macrophage colony-stimulating factor stimulates in vitro mature human neutrophil and eosinophil function, surface receptor expression, and survival. J Clin Invest 1986;78:1220-8.

485. Weisbart RH, Golde DW, Gasson JC. Biosynthetic human GM-CSF modulates the number and affinity of neutrophil f-met-leu-phe receptors. J Immunol 1986;137:3584-7.

486. Weisbart RH, Kwan L, Golde DW, Gasson JC. Human GM-CSF primes neutrophils for enhanced oxidative metabolism in response to the major physiological chemoattractants. Blood 1987;69:18-21.

487. Shikita M, Tsuneoka K, Hagiwara S, Tsurufuji SA.
Granulocyte-macrophage colony-stimulating factor (GM-CSF) produced by carrageenin-induced inflammatory cells of mice.
J Cell Physiol 1981;109:161-9.

488. Warren MK, Ralph P. Macrophage growth factor (CSF-1) stimulates human monocyte production of interferon, tumour necrosis factor and myeloid CSF. J Immunol 1986;137:2281-5.

489. Fleit HB, Rabinovitch M. Interferon induction in marrow-derived macrophages : regulation by L-cell conditioned medium.
J Cell Physiol 1981:108;347-52.

490. Moore RN, Oppenheim JJ, Farrar JJ, Carter CS, Waheed A, Shadduck RK.
Production of lymphocyte-activating factor/interleukin 1 by macrophages activated with colony stimulating factors.
J Immunol1980;125:1302-5.

491. Lin HS, Gordon S. Secretion of plasminogen activator by bone marrow-derived mononuclear phagocytes and its enhancement by colony-stimulating factor. J Exp Med 1979;150:231-45.

492. Nicola NA, Burgess AW, Metcalf D. Similar molecular properties of granulocyte-macrophage colony-stimulating factors produced by different mouse organs in vitro and in vivo.
J Biol Chem 1979;254:5290-9.

493. Morson BC, Dawson IM. Gastrointestinal Pathology, Blackwell Scientific Publications, London, 1979, 551-9.

494. Nicola NA, Metcalf D, Johnson GR, Burgess AW. Separation of functionally distinct human granulocyte-macrophage colony-stimulating factors. Blood 1979;54:614-27.

495. Abboud CN, Brennan JK, Barlow GH, Lichtman MA. Hydrophobic adsorption chromatography of colony-stimulating activities and erythroid-enhancing activity from the human monocyte-like line, GCT. Blood 1981;58:1148-54.

496. Nicola NA, Begley CG, Metcalf D. Identification of the human analogue of a regulator that induces differentiation in murine leukaemic cells. Nature 1985;314:625-8.

497. Peschel C, Paul WE, Ohara J, Green I. Effects of B cell stimulatory factor-1/interleukin 4 on haemopoietic progenitor cells. Blood 1987;80:254-63.

498. Metcalf D. Acute antigen-induced elevation of serum colony stimulating factor (CFS) levels. Immunology 1971;21:427-36.

499. Sheridan JW, Metcalf D. Studies on the bone marrow colony stimulating factor (CSF) : relation of tissue CSF to serum CSF. J Cell Physiol 1972;80:129-140.

500. Pretlow TP, Keith EF, Cryar AK, Bartolucci AA, Pitts AM, Pretlow TG, Kimball PM, Boohaker EA. Eosinophil infiltration of human colonic carcinomas as a prognostic indicator. Cancer Res 1983;43:2997-3000.

501. Werkmeister JA, Phil E, Hughes ES, Cuthbertson AM, Rollo AJ. Lymphoreticular cells within primary colorectal carcinoma : a surface marker study. Clin Immunol Immunopathol 1981;18:415-24.

502. Bland PW, Warren LG. Antigen presentation by epithelial cells of the rat small intestine I. Kinetics, antigen specificity and blocking by anti-Ia antisera. Immunology 1986;58:1-7.

503. Crawford RM, Finbloom DS, Ohara J, Paul WE, Meltzer MS. B cell stimulatory factor-1 (interleukin 4) activates macrophages for increased tumoricidal activity and expression of Ia antigens. J Immunol 1987;139:135-141.